Molecular and ecological analysis of LOX3- and NPR1-dependent defense responses in plant-herbivore interactions

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Manuscript 1- authors' contribution

Using nutritional indices to study *LOX3*-dependent insect resistance

Cbgowda Rayapuram and Ian Baldwin

Plant Cell and Environment, 29: 1585-1594 (2006)

This manuscript describes the effects of *LOX3*-dependent defense responses of *Nicotiana attenuata* on insect nutrition. Under the supervision of Ian T. Baldwin I was responsible for developing the concept of a nutritional analysis assay for insects. I developed the outline and the written version of the manuscript, which was then optimized by Ian T. Baldwin and me.

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Ian T. Baldwin

Manuscript 2- authors' contribution

Increased SA in *NPR1*–silenced plants antagonizes JA and JA-dependent direct and indirect defenses in herbivore-attacked *Nicotiana attenuata* in nature

Cbgowda Rayapuram and Ian Baldwin

Plant Journal, 2007 (in press)

This manuscript describes the effects of NPR1-dependent defense responses of *Nicotiana attenuata* on insect related direct and indirect defenses. Under the supervision of Ian T. Baldwin I was responsible for planning, realization and analysis of all field and glasshouse experiments using the transgenic plants and WT plants. I developed the outline and the written version of the manuscript, which was then optimized by Ian T. Baldwin and me.

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Ian T. Baldwin

Introduction

Introduction to plant defenses

Any organism in order to survive and reproduce successfully has to defend itself from its natural enemies. Autotrophic organisms like plants are always faced with the prospect of being consumed by herbivores, which in turn can have serious consequences for plants' reproductive ability. Fortunately, plants possess a range of evolved traits that help them resist canopy-consuming herbivores. Plants usually rely on mechanical traits to resist herbivores. For example, plants produce resins, lignin, silica, and wax on their epidermis, which can alter the texture of the plant tissue and make it less palatable; physical barriers on the plant's surface (e.g., sharp spines or trichomes) restrict herbivores' movements (Cooper and Smith, 1987). In addition, some plants possess the ability to change their physiology, chemical composition, or even development in response to herbivore attack. These defenses are termed "**induced defenses**" **(IR)**. Induced defense are plastic phenotypic responses that allow plants to mount defenses only at the time when they are most needed (Agarwal, 1999). This can also be viewed as a cost-saving mechanism, since continuous activation of defense response might compromise the allocation of resources that otherwise are needed for growth and reproduction. Plants possess a unique ability to recognize attacking herbivores and can reconfigure their transcriptional responses to produce a diverse set of defense proteins and secondary metabolites: As a part of transcriptional reorganization, genes related to primary and secondary metabolism, photosynthesis, defense, abiotic stress, etc., are differentially regulated (Voelckel and Baldwin 2004). These changes allow the plant to alter its physiology and its chemistry, which can in turn affect the feeding herbivore. The most interesting aspect of any plant-insect interaction is to understand how plants recognize different types of herbivore attack and translate the signals into functional traits.

Role of phytohormones in induced plant defense

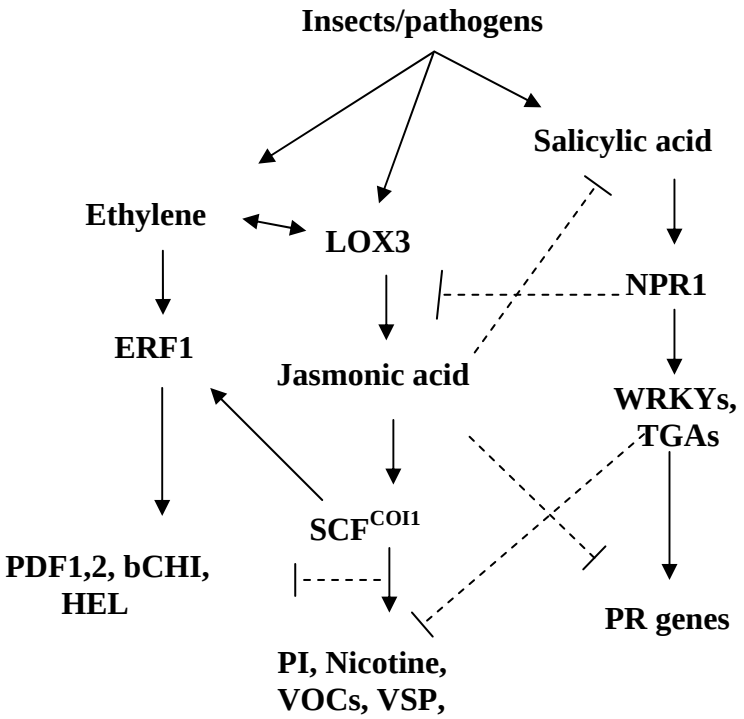
Chemical signals have played a central part in mediating plant responses against abiotic stress and biotic stress (imposed by pathogen and herbivores). In induced resistance (IR), the production of several defense metabolites by plants is to a great extent mediated by phytohormone jasmonic acid (JA) and its dependent signalling responses. These defense

metabolites can affect herbivore growth and development by acting as either anti-feedants, anti-digestion proteins, or growth retardants (Halitschke and Baldwin 2003). Also some

metabolites are produced in the form of volatiles and help the plant attract the herbivore's predator (Turlings, Loughrin et al. 1995; Takabayashi and Dicke 1996; Kessler and Baldwin 2001). Although JA is implicated as a major phytohormone in IR, JA is not the only signal that mediates IR. Rather in response to herbivore damage, plants produce different phytohormones which vary in time and amount. Apart from JA, two other phytohormones, salicylic acid (SA) and ethylene are also implicated in IR. Knowledge of the molecular role of ethylene in IR is emerging (von Dahl and Baldwin, 2007), while the role of SA in IR has not been studied extensively. SA was first associated with plant-pathogen interactions. SA was reported to be vital for inducing pathogenesis-related (PR) proteins and establishing systemic acquired resistance (SAR) (Hunt and Ryals 1996). Later studies reported SA had an additional role in the hypersensitive response (HR- cell death at the site of pathogen infection) (Delaney, Friedrich et al. 1995). Several published studies strongly suggest that SA- and JA-dependent signaling pathways mediate plant defense against pathogens and herbivores respectively. But at the same time, it has become apparent that herbivore or pathogen attack frequently recruits not one but many signal cascades. For example, bacteria (*Pseudomonas syringae*) can activate both the SA and the JA pathways in *Solanum esculentum* (Stout, Fidantsef et al. 1999), while in *Arabidopsis*, herbivore (*Pieris rapae*) damage elicits both JA- and SA-dependent defenses (De Vos, Van Zaanen et al. 2006). The specificity of responses in defense gene expression to particular attackers seems to be the result of a network of interconnecting signal cascades that cross-communicate (Feys and Parker 2000; Glazebrook 2001; Thomma, Penninckx et al. 2001; Heidel and Baldwin 2004). Therefore, the notion that a linear phytohormone-dependent signaling pathway mediates IR seems unlikely. Different phytohormones and their dependent signaling cascades interact (cross-communicate) among themselves to fine-tune a plant's response. Cross-communication among different phytohormones and their dependent signaling cascades provides plants with the regulatory potential that is needed to tailor their responses to the diverse herbivore species that attack them (Walling 2000).

The objectives of my investigation are based on the following observations: **A)** JA is vital amongst all other phytohormones in mediating IR in plants in response to herbivores. **B)** JA-mediated defense metabolites have the ability to affect the growth and development of herbivores and in turn herbivores can counter-respond to plants' defenses by altering their nutritional physiology. **C)** During IR, plants produce high levels of SA in addition to JA and it is likely that the cross-communication between SA and JA to activate IR is indispensable. Therefore, in order to understand the roles of JA and SA as they relate to plant resistance, I

ask two main questions, one from an insect's perspective and the other from a plant's perspective.



Overview of the signal transduction pathways in response to herbivores or pathogens. Jasmonic acid, salicylic acid, and ethylene play crucial roles in any interaction. Synergistic (arrow) and antagonist (dashed and blunted arrow) regulatory mechanisms help the plant regulate the elucidation of a specific defense. In defending against either herbivores or pathogens, *LOX3* and NPR1 are central players, capable of exerting major effects.

I) How *LOX3*-JA-dependent defenses affect insects' nutrition and do insects employ counter-strategies against plants' IR?

II) What role does SA play in a predominantly JA-mediated IR? Do plants coordinate signals from JA and SA pathways to mount a comprehensive IR against herbivores?

***LOX3*-dependent JA signalling cascades and induced defenses: from the plant's perspective**

The phytohormone jasmonic acid (JA) is a linolenic acid-derived oxylipin produced by the octadecanoid pathway; its biosynthetic precursor, 12-oxo-phytodienoic acid

(OPDA), and derivatives, such as its methyl ester (MeJA) or amino acid conjugates, collectively called jasmonates (JAs), belong to the well-characterized class of signals mediating the elicitation of defense responses to herbivory (Creelman and Mullet 1997; Beale and Ward 1998; Blee 1998; Devoto and Turner 2003; Farmer, Almeras et al. 2003; Halitschke and Baldwin 2004). After herbivore attack, plants transiently increase JA production, and increased JA is implicated in activating the transcription of several defense genes (e.g., protease inhibitors and nicotine). *LOX3*, the enzyme that supplies hydroperoxide substrates for JA biosynthesis (Halitschke and Baldwin, 2003), has emerged as a candidate gene that regulates plants' induced defenses by regulating JA biosynthesis. Using powerful transformation techniques such as anti-sense gene expression or RNA-interference (RNAi), a particular gene can be silenced and its function in plant-herbivore interactions studied. One successful example involves *LOX3*. Previous work in our lab have shown that when *LOX3* expression was silenced plants, they accumulated fewer direct and indirect defense metabolites. The impact of *LOX3*-silencing and reduced defense metabolite accumulation is reflected in the behavior of the interacting herbivore, *Manduca sexta*. *LOX3*-silencing rendered plants more susceptible to *M. sexta* (Halitschke and Baldwin, 2003). But the manner in which *LOX3*- and JA-dependent defense metabolites affect herbivore's nutrition (consumption, digestion, and assimilation) is not clear.

LOX3-dependent JA signalling cascades and induced defenses: from herbivore's perspective

We now know a lot regarding the importance of *LOX3*-dependent signalling cascades for producing vital defense metabolites. We also know that *LOX3*-mediated defense compounds negatively affect herbivore growth. Since *LOX3*-dependent JA signaling produces an array of defense metaboilites, it is likely that each of these compounds can affect various growth-related parameters (growth, digestion, food retention and allocation of food to body mass). For example, *LOX3*-dependent protease inhibitors produced by the plants are known to resist insect growth by inhibiting several digestive enzymes present in the insect gut (Zavala, Patankar et al. 2004).

But what has not been known till now are the behavioral counter-responses of the herbivores feeding on plants in which *LOX3*- and JA-dependent defenses are activated. This is vital to understand given that although plants produce blends of defense metabolites, herbivores still manage to complete their life cycle. Therefore, plants can resist an herbivore but cannot eliminate the herbivores completely. It may be that herbivores have learned to deal

with plants' defenses by adapting to the changing nutritional status of the plant. These adaptations seem essential for the herbivores to derive sufficient nutrients amid the several defense compounds. These adaptations can take the form of alterations in behavior and nutritional physiology in response to plants' defenses. Herbivores can learn to change their pre- and post-ingestive behaviors to make the best use of consumed food. Several studies suggest that herbivores have learned to adapt to plants' changing nutritional status using multidimensional suite of physiological and behavioral compensatory responses (Bernays, 1985; Simpson and Simpson, 1989). For example, insects are known to compensate for defenses that decrease the digestibility of leaf proteins (e.g. trypsin proteinase inhibitors, *TPI*s) by increasing consumption rates (Bergelson and Crawley 1992). Insects' digestive tracts are well-adapted organs and are capable of dealing successfully with such protease inhibitors in their diets. For examples, bruchids have adapted to soybean cysteine protease inhibitor by producing both sensitive and insensitive enzymes (Zhu-Salzman, Ahn et al. 2003). With our growing knowledge of the role of *LOX3*-dependent responses in plants' resistance to herbivores, incorporating a detailed nutritional and behavioral analysis of insects feeding on a chemically defended plant will provide meaningful insight into the study of plant-herbivore responses.

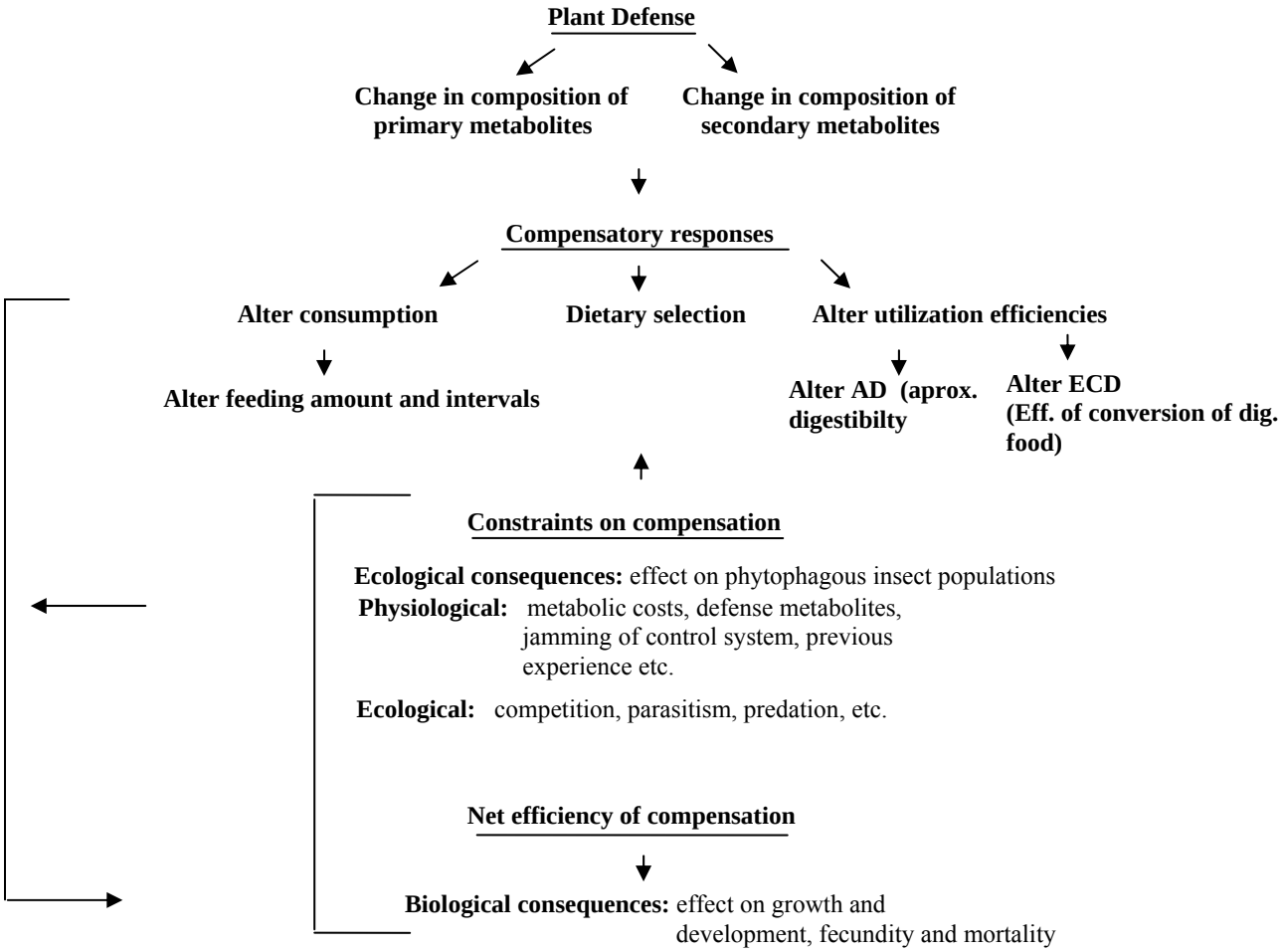
Using nutritional ecology to understand *LOX3*-dependent plant defense

Nutritional ecology measures how efficiently herbivores transform consumed plant material into biomass. Insects have evolved many strategies for doing this. Part of the food that is consumed may not be digested and absorbed. A portion of digested food may be converted to energy and some portion may be converted to insect biomass. Although herbivores can feed on plants whose *LOX3*-dependent defenses have been activated, practically nothing is known about how insects get their nutrition. Herbivores have to withstand plant defense metabolites and at the same time acquire enough nutrients to grow and develop. Part of the energy insects derive from digested food has to go towards detoxifying defense metabolites. Therefore, integrating nutritional ecology in studies related to plant defense will help clarify how different defense metabolites act on insects' physiology.

Tools and strategies to study *LOX3*-dependent effects on herbivores' nutrition

Now that we have highlighted the importance of measuring the digestion, assimilation, and energy allocation in insects feeding on *LOX3*-defense elicited plants, appropriate tools are also needed. Measuring the mass of food larvae ingest, the mass they

gain, and the frass they produce is not enough. We need to know the efficiency of consumption, digestion, and food allocation to obtain a basis for calculating indices which give numerical values to these processes. Such a system was developed by Waldbauer in 1968 to assess the responses of insects feeding on artificial diet. The most frequently used indices measure the mass of leaf consumed (*C*), the body mass gained (*G*), and the mass excreted as frass (*F*) during a particular stage of development.



A diagram summarizing various relationships between the components of nutrition compensation. When herbivores feed on defense-elicited plants they compensate for the altered plant chemistry by increasing their feeding or increasing their AD or ECD. But this compensation is hindered by ecological and physiological factors. The net efficiency of the compensation is therefore determined by the insects' compensation response and the constraints which the herbivore has to deal with.

From these measures and the mass balance equation, $C=G+R+F$, the mass respired as CO_2(R) can be inferred (Waldbauer, 1968). These values can be used to calculate a suite of nutritional indices: CI [(leaf ingested)/(larval mass gain×number of days)] measures the amount of *C* relative to *G* by an insect during the entire feeding period; AD [(leaf mass

ingested−frass mass)/(leaf mass ingested)] measures the efficiency of digestion of ingested food; ECD [(larval mass gain)/(leaf mass ingested−frass mass)] measures the efficiency with which digested food is converted to body mass, and ECI [(larval mass gain)/(leaf mass ingested)] measures the ECI to body mass. These indices have been used to illuminate the pre- and post-ingestive responses of insects to artificial diets of variable quality. Here for the first time we used Waldbauer nutrional assay to study insect counter-responses to *LOX3*- and JA-dependent defenses.

NPR1-dependent SA signalling cascades in plant-herbivore interactions

As stated above, apart from JA, two other phytohormones (SA and ethylene) are also thought to play a vital role in induced plant resistance to herbivores. The role of ethylene in *N. attenuata* has been studied to some extent (von Dahl, 2007), but how SA mediates herbivore resistance is not clearly understood. SA is known to play an important role in plant-pathogen interactions and to be vital for inducing pathogen resistance. Advances in molecular biology have shown that plants can activate multiple pathways simultaneously (Glazebrook, Chen et al. 2003), making the notion of linear pathways involved in herbivore or pathogen resistance outdated. As a part of their induced resistance to herbivores, plants stimulate JA (Halitschke and Baldwin, 2004) and SA (Stotz, Koch et al. 2002; Heidel and Baldwin 2004). Although plants activate a JA-based signalling pathway in response to herbivores, the involvement of other (SA and SA-dependent) pathways cannot be ruled out. Different phytohormone-dependent pathways function as a network, and an extensive crosstalk between different pathways determines each specific response. As *LOX3* is an important component of JA-dependent signal cascades, the non-expressor of *PR-1* (NPR1) is important for SA signalling cascades; induced resistance to pathogens is known as “systemic acquired resistance” (SAR). NPR1 is activated by SA and after activation acts as a transcription activator. NPR1 binds to transcription factors called TGAs and induces pathogenesis-related (PR) defense genes (Zhang, Fan et al. 1999). Several PR proteins possess antimicrobial characteristics and are thought to contribute to resistance in plants. NPR1 is thought to mediate the antagonism between SA and JA pathways; these are known to be mutually antagonistic (Spoel, Koornneef et al. 2003). A plant infected with a pathogen resists the pathogen but also becomes susceptible to herbivores (Stotz et al. 2002). This is because the NPR1-dependent SA signaling cascades inhibit JA defense responses. However, this does not always occur. For example, necrotropic bacteria (*Pseudomonas syringae*) can activate both the SA and JA pathway in *Lycopersicon esculentum* (Stout, Fidantsef et al. 1999).The JA and

SA pathways in this case act synergistically and as a result, local infection by *P. syringae* causes systemic resistance to a noctid moth (*Helicoverpa zea*). The second part of my study tries to answer how the SA-based signal cascade mediates plant-herbivore interactions and how these signals interact with JA-based signalling pathway.

Understanding the role of NPR1 in induced plant defenses

The non-expressor of *PR-1* (NPR1) is known to be a major molecular player in SAR. NPR1 functions as a transducer of SA, which is produced after pathogen attack. NPR1 was first identified in *Arabidopsis* in genetic screens for SAR-compromised mutants (Cao, Bowling et al. 1994; Delaney, Friedrich et al. 1995). Pathogen attack results in changes in cytosolic cellular redox as well as increases in the levels of SA; these increases cause the constitutively present NPR1 protein to de-polymerize and form monomers (Mou, Fan et al. 2003) which migrate to the nucleus where they associate with transcription factors (TGA family) that induce pathogenesis-related (*PR)* defense genes (Zhang et al. 1999). However, NPR1's function is not restricted to SA-dependent responses; NPR1 also interacts with different signaling cascades in response to different attackers. For example, during induced systemic resistance (ISR: a biologically elicited, systemic defense response activated when roots are colonized by particular strains of non-pathogenic rhizobacteria), JA and ethylene increases are mediated via NPR1, independently of SA (Pieterse and Van Loon 2004). Among other functions, NPR1 can negatively regulate SA biosynthesis during pathogen attack (Shah 2003). Recently, NPR1 has been shown to mediate the SA-induced suppression of JA-dependent responses (Spoel, Koornneef et al. 2003). These studies have highlighted the diverse roles that NPR1 plays in plants. The *NPR1* gene encodes a protein with a BTB/BOZ domain and an ankyrin-repeat domain; both domains are characteristic of proteins with diverse functions (Bork 1993; Cao, Glazebrook et al. 1997; Aravind and Koonin 1999).

Strategies and tools

We transformed *N. attenuata* plants with an RNAi construct harboring a fragment of Na-*NPR1* in an inverted repeat orientation (ir-*npr1*) to silence expression of the endogenous *NPR1* gene. We compared the performance of larvae of the second-most important native lepidopteran herbivore of *N. attenuata*, *Spodoptera exigua,* on ir-*npr1* and WT lines in experiments conducted in the glasshouse as well on native herbivores in the plant's native habitat, the Great Basin Desert. To understand the resistance phenotypes, we measured the production of different phytohormones (total SA, free SA, conjugated SA, JA,

JA-amino acid conjugates), direct defense metabolites (nicotine, rutin, and caffeoyl putrescine), indirect defense metabolites (VOCs), and gene expression profiles. The results demonstrate that Na-NPR1 and its associated phytohormone, SA, influence JA-dependent IR and in doing so influence both direct and indirect defenses.

Introducing the model system

The plant model system I currently work with is *Nicotiana attenuata*, an annual native to southwestern USA. *N. attenuata* germinates from dormant seed banks in response to factors in smoke following fires in the desert habitat. The nutrient-rich soil that is found after the burns facilitates the growth of many competitors of *N. attenuata* and also of new populations of the different herbivores and pathogens which re-colonize the area. *N. attenuata* (2n=24), a solanaceous plant, is self-pollinated with occasional cross-pollination occurring. What makes *N. attenuata* an interesting model system is its ability to produce a wide range of direct defense metabolites (for example, nicotine, proteinase inhibitors), and indirect defense metabolites in the form of volatile organic compounds (cis-α-bergamotene). *N. attenuata* attracts more than 20 types of herbivores. The most extensively studied herbivore is *Manduca sexta* (tobacco hornworm), a known specialist on *N. attenuata* and resistant to nicotine.

A) Elongated and flowering plant of *Nicotiana attenuata* on the burnt soil in its natural habitat. **B)** Herbivores of *N. attenuata* (clockwise) *Manduca sexta, Emboasca sps, Sylvilagus sps, Ephitrix sps, Spodoptera exigua, Tupiocoris notatus, Diabrotica sps.* **C)** Predator of *M. sexta* eggs *Geocoris pallens*

I studied the effects of *N. attenuata*'s *LOX3*-dependent signalling cascade on the various nutritional aspects of the specialist herbivore *M. sexta*. From a plant's point of view, *LOX3* defense has been well characterized (Halitschke and Baldwin, 2003) and the details of the metabolites that *LOX3* influences in a plant are well known. But how these effects are

transformed into insect nutrition is not known. Here, for the first time, the Waldbauer assay is used on genetically modified plants to compare the changes in nutritional indices of larvae feeding on WT plants and *LOX3*-silenced plants (Manuscript I).

In addition, I studied how NPR1 influences induced resistance in *N. attenuata*. NPR1's function in pathogen resistance has been well studied but not much is known regarding its involvement in induced herbivore resistance. The rationale behind this study was the transient increase in *NPR1* and SA levels after herbivory in *N. attenuata*, which prompted us to speculate that NPR1 has a functional role during IR. We carried out this study under glasshouse and natural conditions in order to understand NPR1's ecological relevance (Manuscript II).

Manuscript 1

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Using nutritional indices to study LOX3-dependent insect resistance

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Key-words: Nicotiana attenuata; jasmonate signalling; Manduca sexta
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Abbreviations:
AD, approximate digestibility;
as-lox, plants silenced in NaLOX3 expression;
CI, consumption index;
ECD, efficiency of conversion of digested food;
ECI, efficiency of conversion of ingested food; ET, ethylene;
JA, jasmonic acid;
NaLOX3, lipoxygenase 3;
PR-1, pathogenesis-related gene-1;
R, Manduca sexta regurgitant;
SA, salicylic acid;
SAR, systemic acquired resistance;
TPI, trypsin proteinase inhibitor;
VIGS, virus-induced genesilencing;
W, wounding.

Running title: *LOX3* in herbivore resistance

Abstract

Induced resistance to biotic attackers is thought to be mediated by responses elicited by jasmonic acid (JA), a subset of which are lipoxygenase 3 (LOX3) dependent. To understand the importance of LOX3-mediated insect resistance, we analysed the performance of *Manduca sexta* larvae on wild-type (WT) and on isogenic *Nicotiana attenuata* plants silenced in NaLOX3 expression and JA signalling, and we used Waldbauer nutritional indices to measure the pre- and post-ingestive effects. LOX3-mediated defenses reduced larval growth, consumption and frass production. These defenses reduced how efficiently late-instar larvae converted digested food to body mass (ECD). In contrast, LOX3-mediated defenses decreased approximate digestibility (AD) in early instar larvae without affecting the ECD and total food consumption. Larvae of all instars feeding on defended WT plants behaviourally compensate for their reduced body mass by consuming more food per unit of body mass gain. We suggest that larvae feeding on plants silenced in NaLOX3 expression (*as-lox*) initially increase their AD, which in turn enables them to consume more food in the later stages and consequently, to increase their ECD and efficiency of conversion of ingested food (ECI). We conclude that *N. attenuata*'s oxylipin-mediated defenses are important for resisting attack from *M. sexta* larvae, and that Waldbauer nutritional assays reveal behavioural and physiological counter responses of insects to these plant defenses.

Introduction

Plants resist attack from insects and pathogens in nature with constitutive and induced defenses. Different phytohormones function as signals that coordinate defense responses among cells, tissues, and organs to resist invading pathogens or insects. Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are three of the best studied, and they influence each other through a complex network of regulatory interactions (Reymond and Farmer, 1998; Walling, 2000; Kessler and Baldwin, 2002). The JA- and SA-signaling pathways elicit suites of genes, phytochemicals, and proteins, which are thought to be specialized against insects and pathogens, respectively, and subject to extensive cross-talk and mutual inhibitory effects (Felton and Korth, 2000; Thaler et al. 2002). The methylated forms of SA and JA (MeSA and MeJA) and the functional analogues of SA {BTH and INA (2,6-dichloroisonicotinic acid)} and JA {indanone-Ile-ME (indanone-isoleucine methyl ester) and coronatine} are commonly used to elicit JA and SA signaling and the respective reporter genes for these processes (e.g., PDF1.2, PR-1) (Lawton et al. 1995; Tally et al. 1999; Thaler et al. 2002; Heidel and Baldwin, 2004; and Schüler et al. 2004). Yet it is still not clear how well these elicitors mimic the plant's responses to insect and pathogen attack. For example, a 789-gene oligomicroarray analysis of *Nicotiana attenuata* responses revealed that the putative SA mimic BTH elicited both SA- and JA-elicited genes (Heidel and Baldwin, 2004). A more informative way to evaluate the importance of SA and JA signaling for insect resistance is to genetically manipulate the endogenous production or perception of the signals and measure the consequences of altered signaling for herbivore performance.

Studies that have examined the performance of pathogens and insects on plants genetically deficient in SA or JA signaling report a more complex view of signaling cross-talk. Studies with mutants defective in JA production have demonstrated that JA signaling mediates resistance to both insect (Halitschke and Baldwin, 2005) and pathogen attack (Thomma et al. 1998; Pieterse and Loon, 2004). A JA-non-perceptive mutant *coi1* in the same study failed to resist the herbivore, suggesting the importance of JA in insect resistance (Stotz et al. 2002). A constitutive JA-signaling-activated mutant *cev1* displays enhanced resistance to aphids, fungal, and bacterial pathogens (Ellis, Karafyllidis & Turner 2002). Greater susceptibility to noctuid larvae, thrips, and spider mites was observed in the tomato mutant *spr1* that had been blocked in its wounding and systemin-signaling processes, which otherwise are required to induce JA synthesis (Howe and Ryan, 1999). These studies demonstrate the value of genetic manipulation as a tool for studying the effects of JA-mediated responses on insect performance.

The analysis of insect performance as a response variable in studies with signaling mutants deserves additional discussion. Inferences about insect performance are based on diverse measures: the percentage of leaf area consumed (Stotz et al. 2002); mass gain and leaf area consumed (Mewis et al. 2005); survival rates (Musser, 2002); number of bites (Bartlet et al. 1999); fecundity (Moran and Thompson, 2001); and host plant choice (Steppuhn et al. 2004). A plant's resistance to insects is a complex, multidimensional process in which different direct and indirect defenses are elicited by different pathways (Kessler and Baldwin, 2004). Insects must counter these defenses with a similarly multidimensional suite of physiological and behavioral compensatory responses (Bernays, 1985; Simpson and Simpson, 1989). For example, insects are known to compensate for defenses that decrease the digestibility of leaf proteins (e.g., proteinase inhibitors: TPIs) by increasing consumption rates (Bergelson and Crawley, 1992) as well as by secreting PI-insensitive proteases (Jongsma and Bolter, 1997). Entomologists have long recognized the challenges that phytophagous insects face in consuming food that is nutritionally dissimilar from the composition of their own tissues and have developed techniques for characterizing the pre- and post-ingestive consequences of variations in food quality for insect growth.

The most frequently used techniques are indices that measure the mass of leaf consumed (C), the body mass gained (G), and the mass excreted as frass (F) during a particular stage of development. From these measures and the mass balance equation, $C = G + R + F$, the mass respired as CO_2 (R) can be inferred (Waldbauer, 1968). These values can be used to calculate a suite of nutritional indices: consumption index (CI) = (leaf ingested)/

(larval mass gain* number of days) measures the amount of leaf consumed relative to the mass gained by an insect during the entire feeding period; approximate digestibility (AD) = (leaf mass ingested – frass mass)/ (leaf mass ingested) measures the efficiency of digestion of ingested food; efficiency of conversion of digested food (ECD) = (larval mass gain)/ (leaf mass ingested – frass mass) measures the efficiency with which digested food is converted to body mass and efficiency of conversion of ingested food (ECI) = (larval mass gain)/ (leaf mass ingested) measures the efficiency of conversion of ingested food to body mass. These indices have been used to illuminate the pre- and post-ingestive responses of insects to artificial diets of variable quality (Dadd, 1985; Simpson & Simpson, 1989; Slansky, 1993) as well as the responses of plants to changes in atmospheric CO_2 concentrations (Whittaker, 1999; Fuhrer, 2003) and to foliar JA and SA sprays (Thaler et al. 2002). We are unaware of any study that has conducted a detailed Waldbauer analysis on an insect feeding on plants whose SA- or JA-signaling abilities have been genetically altered.

We use Waldbauer nutritional assays to analyze the importance of LOX3-mediated defenses against the larvae of the solanaceous specialist, *Manduca sexta*. We use isogenic lines of *Nicotiana attenuata* that differ only in oxylipin signaling due to the antisense expression of NaLOX3, the enzyme that supplies hydroperoxide substrates for JA biosynthesis (Halitschke and Baldwin, 2003). We measure transcripts of NaLOX3 and reporter genes for JA signaling (TPI) and for SA signaling (PR-1). To accurately measure the mass of leaf material consumed and facilitate the measurements of frass produced, we use an excised leaf assay and confirm the changes in body mass with independent replicates performed on intact plants.

Materials and Methods

Plant material

WT *N. attenuata* plants selfed for 14 generations (seeds derived from a collection from a native population from DI Ranch, Santa Clara, UT, USA) and line A300, in which NaLOX3 is expressed in an antisense orientation in the WT genotype, as characterized in Halitschke and Baldwin (2003), were used in the experiment. Germination was carried out according to the procedures described by Krügel et al. (2002). After 10 days seedlings were transferred to 1 L pots.

Isolation of PR-1

Primers to amplify PR-1 (PR1N FP (5'CGATTGCCTTCATTTCTTCTTGT 3' and PR-1N RP (5'GTCGTCCCAGGTTAAAGGTTCTAC3') in *N. attenuata* were designed based on the published sequences from *Nicotiana tabacum* coding sequence. Total RNA was extracted as described in the TRI reagent protocol (Sigma, Taufkirchen, Germany) from plants elicited with W+R and W+W, treatments and cDNA was synthesized using an invitrogen SuperScript cDNA synthesis kit (California, USA). A 148 bp PR-1 fragment was PCR amplified using the primers listed above. The fragments were excised from the gel, purified using an Amersham gel purification kit (Buckinghamshire, UK), and cloned in a pGEM-T Easy vector (Promega, Madison, WI, USA) following the manufacturer's instructions. Positive colonies were picked by blue-white screening and grown overnight, and the plasmid was isolated with a Macheray and Nagel kit (Duren, Germany). The fragments were sequenced and confirmed as PR-1 after being compared with the known sequences from the NCBI database.

VIGS inoculation

N. attenuata seeds from a greenhouse-grown fourteenth-generation collection originating in Utah (Baldwin, 1998) were germinated as described above. All plants were grown under a 32/27 °C 16/8 h light/dark regime until they were 3-4 weeks old and in the early rosette stage of growth. A culture of the pTV00 (empty vector) plasmid containing *A. tumefaciens* was grown overnight in a media containing 50 mL YEP with 50mg/L of kanamycin as a selectable marker at 28° C and 200 rpm. The culture was grown until it attained an OD_{600} of 0.4-0.6, at which time the cells were centrifuged at 4000 rpm at 4°C for 10 minutes. After the supernatant was discarded, the pellet was resuspended in 5 ml of 10mM $MgCl_2$ and 5 ml of 10 mM MES. A 5 ml inoculation solution was used to infect the abaxial surface of 3 leaves per plant using a syringe without the needle. Plants were enclosed in plastic bags in the dark for 2 days at 22° C at 65% relative humidity. After 2 days the light levels were returned to normal (400-1000 μmol m^{-2} s^{-1}).

Nucleic acid analysis

Total RNA was extracted following the TRI reagent method (Sigma, Taufkirchen, Germany), and checked for purity and DNA contamination by carrying out PCR with all the primers with 100 ng of RNA as template. 1μg of the total RNA was reverse transcribed to cDNA using a SuperScript cDNA synthesis kit (California, USA). To determine whether equal quantities of the cDNA were produced, we conducted a short PCR of

18 cycles with ECI primers (a constitutively expressed gene) for all samples. We used the following primers to analyze the expression of LOX3, PI, and PR-1: LOX3 forward 5' GGCAGTGAAATTCAAAGTAAGAGC 3' LOX3 reverse 5' CCCAAAATTTGAATCCACAACA 3' amplifying a region of 271bp; PR-1 PR1N FP 5' CGATTGCCTTCATTTCTTCTTGT 3', PR1N RP 5' GTAGAACCTTTAACCTGGGACGAC 3' amplifying a region of 148 bp; PI-03 5'GGCTGTTCACAGAGTTAGCTTCCTTG 3', PI-04 5' GCTCCACTGCCATATTACAGATTACAGGC 3' amplifying 1.45 kb fragment and ECI ECI FP 5'AGAAACTGCAGGGTACTGTTGG3', ECI RP 5'CAAGGAGGTATAACTGGTGCCC3' amplifying a region of 137 bp. The LOX3 primers were designed in such a way that they amplified the endogenous gene but not the transgene. PCR conditions were optimized, and 20 cycles of amplifications were selected for ECI, LOX3, and PI, and 26 cycles for PR-1. LOX3, PI, PR-1 and ECI genes were PCR amplified from 100 ng of cDNA in all three biological replicates from each of the different VIGS treatments [WT ev (empty-vector inoculation of WT plants); *as-lox* ev (empty-vector inoculation of NaLOX3-antisense plants)]. Band fluorescence was scored using a Syngene Gene Tools Version 3:00:22 bio documentation apparatus (Cambridge, UK) and quantified relative to ECI gene fluorescence.

Insect performance assays

Single leaves from 15 plants from each of the VIGS experiments were wounded with a fabric pattern wheel, after which the puncture wounds were immediately treated with either 20 µL of distilled water (W+W) or 1:1 water diluted regurgitant (W+R) from *M. sexta* larvae that has been collected previously. After 48 h another leaf occupying the next adjacent node opposite to the elicited leaf was elicited in the same way. This process was repeated for a total of 6 elicitations over 11 days. Twenty-four hours after the first elicitation, 100 mg leaf material without midribs was excised from each of the 15 elicited plants from each treatment group and placed into 15 small round plastic containers (3.5cm x 4.5cm ht:diameter) (DG-Distler-Gastro Gmbh, Erfurt, Germany), each housing a neonate *M. sexta* larvae. The boxes were closed with perforated plastic lids, placed in an incubator, and maintained at 75% humidity, 26°C with a 16:8 h light:dark cycle. Larvae were supplied with 100 mg of fresh leaf material that had been elicited 24 h previously by W+R treatments once every 48 h for three times. From the fourth feeding onwards, the mass of supplied leaf material was increased to 200 mg so that the larvae were never without food.

The Waldbauer analysis was conducted over an 11-day experimental period during which larvae were supplied with leaf material 5 times. At the end of 11 days, total dry mass of insects, remaining leaf, and frass egested was recorded after being dried at 65° C for three days; after which a constant dry mass was obtained. Consumption index (CI) = (leaf mass ingested)/ (larval mass gain* number of days), approximate digestibility (AD) = (leaf mass ingested – frass mass)/ (leaf mass ingested), efficiency of conversion of digested food (ECD) = (larval mass gain)/ (leaf mass ingested – frass mass), and efficiency of conversion of ingested food (ECI) = (larval mass gain)/ (leaf mass ingested)} were calculated (Waldbauer, 1968). A discussion of the sources of errors in Waldbauer assays and how these errors were addressed in these trials can be found in the supplementary materials (Supplemental Material). One-way ANOVAs with Bonferroni-corrected post-hoc tests were used to analyze the data.

Results

LOX3, TPI, and PRI transcripts

A quantitative RT-PCR on three R-elicited plants from empty-vector inoculations of WT and NaLOX3-antisense plants (*as-lox* ev) was carried out to confirm that LOX3 was silenced in the *as-lox* transgenic plants and that the VIGS experiments do not alter the reduced endogenous NaLOX3 transcript levels in the *as-lox* stable transformed lines. LOX3 transcript accumulation differed significantly between WT ev and *as-lox* ev (ANOVA, $F_{1,\ 4}$ 104.96, $p < 0.001$, Fig. 1A, B). Significantly fewer NaLOX3 transcripts accumulated in *as-lox* ev than in WT ev. This observation suggests that LOX3 was silenced in the *as-lox* transgenic plants and that NaLOX3 silencing was not affected by the VIGS experiments.

To evaluate the effect of LOX3 silencing on downstream defense gene expression, we analyzed transcripts of trypsin protease inhibitor (TPI) and pathogenesis-related protein-1 (PR-1) 12 h after eliciting leaves by wounding and treating the wounds with regurgitant (R) from *M. sexta* (W+R). TPI was chosen as a reporter for defense genes elicited by the LOX3-dependent JA pathway and PR-1 was used as a reporter for the SA dependent/independent pathway. TPI transcripts, which differed significantly between WT ev and *as-lox* ev (ANOVA, $F_{1,\ 4}$ 37.97, $p < 0.003$, Fig. 1A, B), tracked LOX3 expression. Significantly fewer (52%) TPI transcripts accumulated in the LOX3-silenced plants (*as-lox* ev) compared to WT ev ($p< 0.001$, Fig. 1A, B) plants. Interestingly, W+R treatment induced PR-1 transcript

accumulation in WT ev as well as *as-lox* ev but the differences were not significant (ANOVA, $F_{1,4}$ 0.356, p = 0.581, Fig. 1A, B). This observation suggests that factors in the oral secretion when applied to wounded leaves of *N. attenuata* trigger LOX3 which in turn influences TPI gene expression in a more or less linear manner.

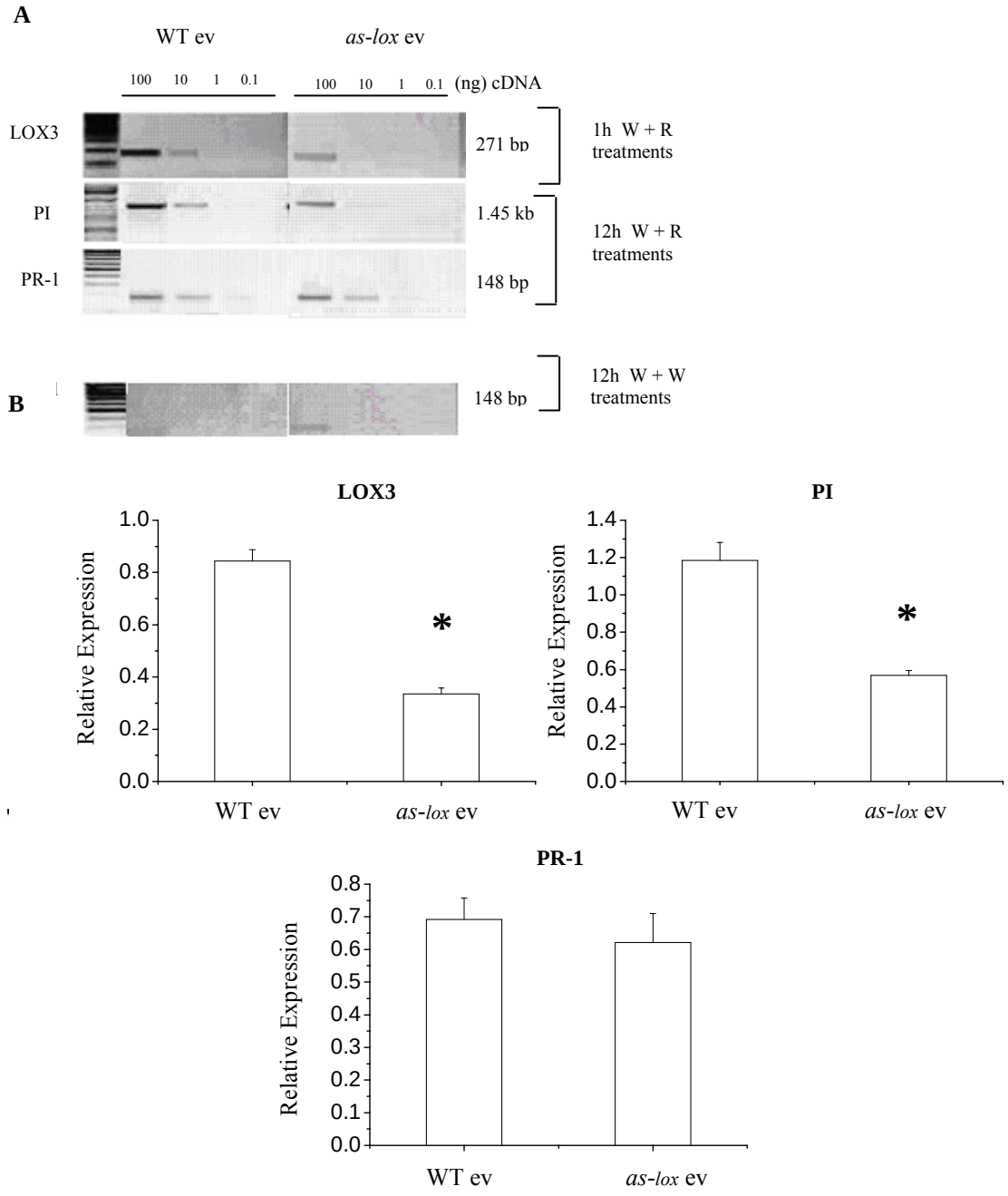

Figure 1. A) Gene expression analysis of LOX3, PI, and PR-1 in WT and *as-lox N. attenuata* plants that were inoculated with *Agrobacterium*-harboring TRV constructs containing an empty vector (ev) and elicited by a wound + regurgitant (W+R) treatment. A 1-hour sample was used to study LOX3 gene expression, and a 12-hour sample was used to study PI and PR-1 gene expression. Below, in the same

panel, is the PR-1 gene expression profile in wound + water (W+W) treated plants after 12 hours. None of the plants showed differential PR-1 gene expression.

B) Quantitative gene expression of LOX3, PI, and PR-1. Mean (±SE) PCR-amplified gene specific band intensity from 100 ng cDNA from each VIGS treatment expressed relative to the band intensity of a PCR-amplified constitutively expressed gene (ECI) from 10 ng cDNA from the corresponding VIGS treatment. Different letters indicate significant differences between treatments by one-way ANOVA. See Supplemental Fig. S1 for loading controls.

LOX3-mediated resistance to *M. sexta* larvae

Waldbauer nutritional indicies were used to study LOX3-mediated signaling on larval performance. The means (+/- SE) of each nutritional index are summarized in Supplemental Table 1. Mass gain among larvae feeding on induced and excised leaves of WT ev and *as-lox* ev was significantly different (ANOVA, $F_{1, 16}$ 16.79, $p < 0.001$, Fig. 2B). The effect of LOX3-derived signaling on larval mass was very dramatic: larvae that fed on *as-lox* ev were 244% larger than those that fed on WT ev plants. Clearly, LOX3-derived signals play a crucial role in determining the performance of *M. sexta* larvae on *N. attenuata*.

LOX3-mediated defenses decrease food intake and efficiency of allocation of energy to body mass

An increase in food consumption was largely responsible for the dramatic increases in body mass gain in larvae that fed on LOX3-silenced plants (*as-lox* ev) (ANOVA, $F_{1, 4}$ 7.74, $p = 0.013$, Fig. 2C). In comparison to larvae feeding on WT ev plants, larvae that fed on *as-lox* ev plants consumed 72% more leaf mass. The ECI was significantly higher in larvae that fed on *as-lox* ev (106%) (ANOVA, $F_{1, 4}$ 11.41, $p = 0.003$, Fig. 2C) when compared with those that fed on WT ev. In contrast to ECI, the CI was significantly lower in larvae that fed on *as-lox* ev (55%) (ANOVA, $F_{1, 4}$ 11.58, $p = 0.003$, Fig. 2C) than in larvae that fed on WT ev. Interestingly, AD did not differ between larvae that fed on *as-lox* ev (ANOVA, $F_{1, 4}$ 0.309, $p = 0.585$, Fig. 2C) and those that fed on WT ev. These results demonstrate that apart from reducing ECI and increasing CI, LOX3-mediated signaling causes larvae to consume significantly less but does not influence the AD of the leaf material. Hence the dramatic increase in the mass of larvae feeding on LOX3-silenced plants can clearly be attributed to increased food consumption.

Decreased food consumption and a reduced ECD: characteristic features of LOX3-mediated signaling

The nearly two-fold increase in the body mass of larvae that fed on LOX3-silenced plants can be ascribed to increased food intake. This increase in food intake can be linked to increased larval growth because the larvae feeding on *as-lox* ev have a higher ECI (gross growth efficiency) than those feeding on WT ev. We wondered if increased leaf intake is associated with a post-ingestive nutritional change that eventually could account for the increased ECI in larvae feeding on leaves excised from *as-lox* ev plants. Interestingly, higher leaf intake was not associated with increased approximate digestibility (AD), as the frass egested clearly related to total leaf consumption by larvae feeding on *as-lox* ev plants. On the contrary to AD, we found that ECD (efficiency of conversion of digested food), which measures the capacity of larvae to convert digested food to body mass, was significantly higher (70%) (ANOVA, $F_{1,\ 4}$ 7.17, p = 0.016, Fig. 2C) in larvae feeding on *as-lox* ev compared to those feeding on WT ev. Since TPI transcripts were also significantly reduced in *as-lox* ev plants compared to WT ev (ANOVA, $F_{1,\ 4}$ 37.97, p = 0.003, Fig. 1), it could well be that a higher TPI activity would result in a reduced ECD and ECI in larvae feeding on WT ev.

Reduced AD in early instars by LOX3 leads to decreased leaf intake in later instars

M. sexta larvae reared at 26ºC typically reach the third larval instar in 11 days when they feed on WT plants. Since the nutritional indices were determined over the entire 11-day experiment, changes in performance that occur during the third instar will dominate those that occur during the first and second instars due to the large size and appetites of later-stage larvae (Lindroth, 1993). So that our analysis would be more sensitive to the responses of earlier-stage larvae, we reanalyzed the responses of larvae that fed on WT and *as-lox* plants over 7 days, the time when larvae that fed on WT plants would just be finishing their second instar. Larvae feeding on induced *as-lox* and WT type *N. attenuata* plants ingested the same amount of leaf material (ANOVA, $F_{1,20}$ 2.426, p = 0.135, Fig. 4B) and had the same ECD (ANOVA, $F_{1,20}$ 0.153, p = 0.699, Fig. 5C).

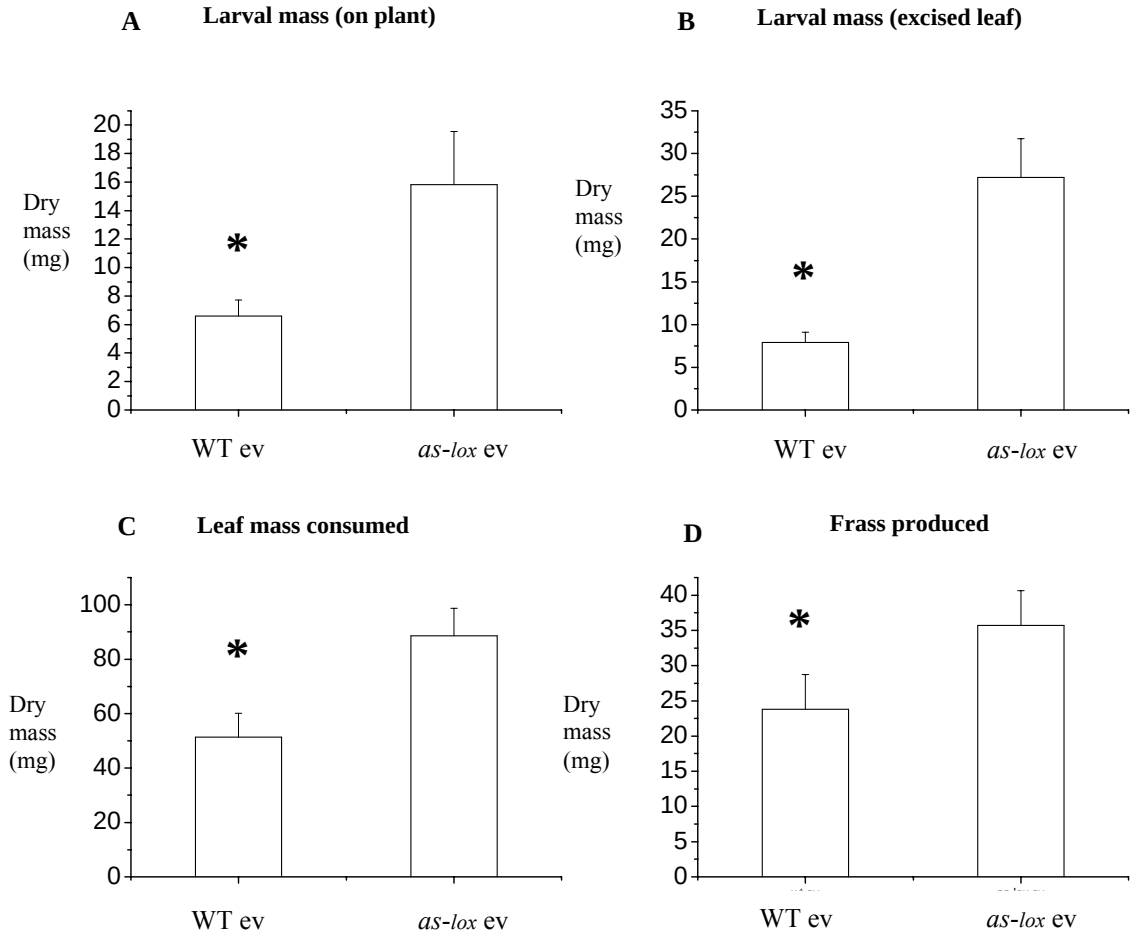

Figure 2. Mean (± SE) primary nutritional indices after 11 days of larvae feeding on WT or *as-lox N. attenuata* plants that were previously inoculated with *Agrobacterium*-harboring TRV constructs containing an empty vector (ev) and elicited with a wound plus regurigitant treatment (W+R) treatment. **A**) Larval dry mass after feeding on induced plants; **B**) larval dry mass after feeding on excised leaves; **C**) total leaf consumed; and **D**) total frass egested. Asterisk indicates significant differences between treatments as determined by one-way ANOVA ($p < 0.05$).

Interestingly, AD was significantly higher in larvae that fed on *as-lox* plants compared to those that fed on WT plants in a 7-day assay (ANOVA, $F_{1,20}$ 6.406, p = 0.019, Fig. 5B), which was not the case in the 11-day assay. A higher AD in larvae that fed on *as-lox* plants was probably due to the lower amount of frass produced by these larvae (ANOVA, $F_{1,20}$ 14.196, p = 0.002, Fig. 4A).

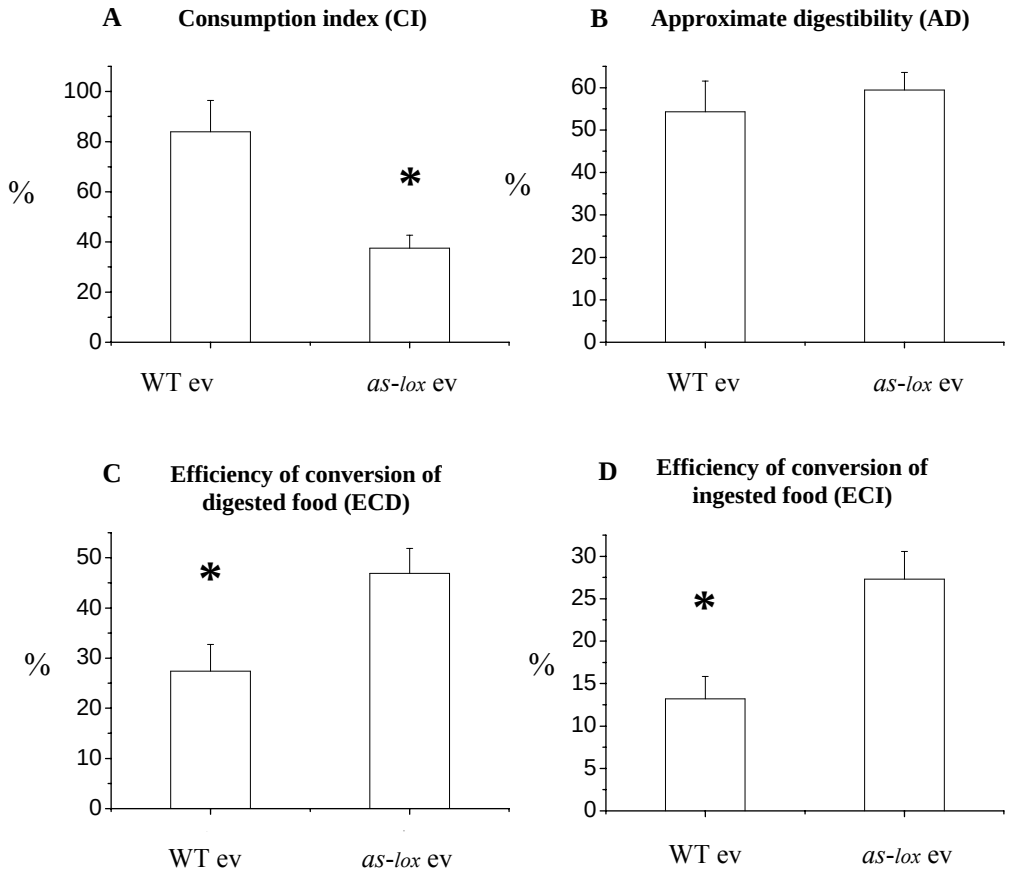

Figure 3. Mean (± SE) nutritional indices after 11 days of larvae feeding on WT or *as-lox Nicotiana attenuata* plants that were previously inoculated with *Agrobacterium*-harboring TRV constructs containing an empty vector (ev) and elicited by a W+R treatment. **A**) consumption index (CI), **B**) approximate digestibility (AD), **C**) efficiency of conversion of digested food (ECD), and **D**) efficiency of conversion of ingested food (ECI). Asterisk indicates significant differences between treatments by one-way ANOVA ($p < 0.05$).

These observations demonstrate that the initial growth differences in the first- and second-instar larvae feeding on WT and *as-lox* plants can be attributed to changes in AD but not ECD. Collectively, the results of the two experiments suggest that larvae feeding on *as-lox* plants initially increase their AD, which in turn enables these larvae to consume more food in the later stages and consequently to increase their ECD and ECI.

Discussion

We conducted a Waldbauer nutritional assay of *M. sexta* larvae feeding on *Nicotiana attenuata* plants that are reduced in LOX3 transcripts, which are known to be involved in JA-signaling pathways. We hypothesized that plants respond to factors present in the regurgitant

of *M. sexta* mainly by activating JA pathways, which in turn are capable of affecting one or more nutritional indices in insects.

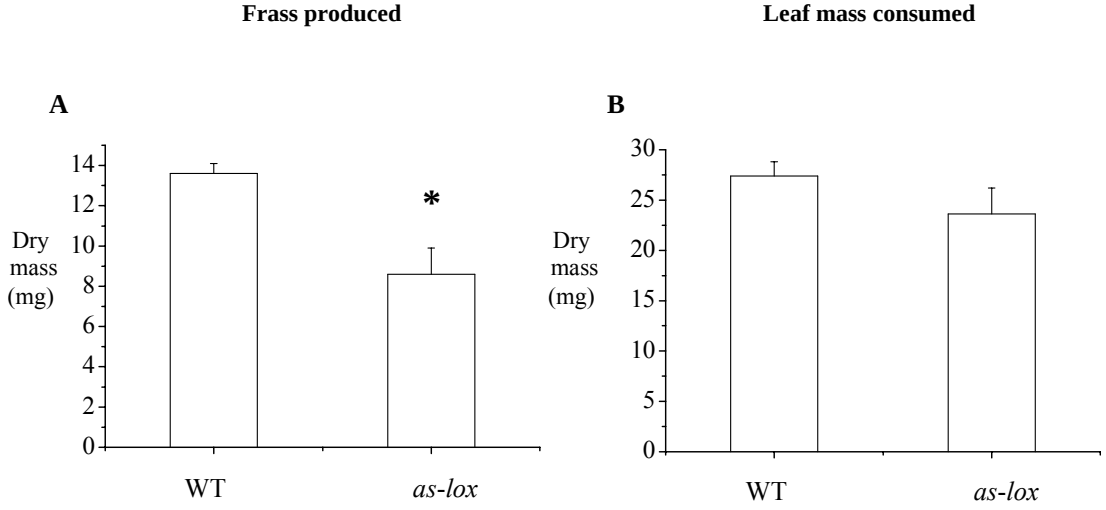

Figure 4. Mean (± SE) primary nutritional indices in larvae feeding on W+R elicited *as-lox* (LOX-silenced plants) and WT *Nicotiana attenuata* plants after 7 days of feeding. **A**) frass egested, **B**) total leaf consumed. Asterisk indicates the level of significant differences between the two genotypes ($p < 0.05$).

Regurgitant (R) elicitation increases LOX3 transcript accumulation in WT plants. Using the Waldbauer analysis we demonstrated that LOX3 controlled the expression of traits that affected larval performance. The LOX3-mediated traits dramatically influenced larval mass by reducing both leaf intake and the efficient conversion of digested food to body mass (ECD), without changing the approximate digestibility (AD) of the leaf material consumed when measured in an 11-day trial. Larvae attempt to compensate for reduced ECI by increasing their consumption index (CI), a response that has been described in many nutritionally impaired insects (Price et al. 1980). Similar experiments conducted over a short period (7 days) demonstrated that LOX3-dependent defense responses do not affect total food consumption but do affect frass production significantly. This observation points to the role of LOX3 in altering AD in early-instar larvae. A reduced AD forces larvae feeding on WT plants to reduce their total food consumption as they move into the later instars in an effort to increase their digestion efficiency.

Responses regulated by LOX3

LOX3-mediated JA signaling accounts for a major part of induced resistance when plants are damaged by insect herbivores. LOX3-mediated JA signaling is known to increase accumulation of several defense-related compounds such as PI and polyphenol oxidase (PPO) (Howe et al. 1999; Thaler et al. 2002; Li et al. 2004), and nicotine (Halitschke and Baldwin, 2003), which are capable of slowing larval growth. Other compounds, such as phenolic acids (Summers and Felton, 1994), phytoecdysteroids (Schmelz et al. 1999), and several genes that encode enzymes involved in terpenoid metabolism (Litvak and Monson, 1998), increase in insect-attacked plants. Silencing LOX3 severely reduced TPI transcripts and larvae that fed on leaves from such plants gained considerably more body mass than did those that fed on WT leaves. TPI slows the growth of insects by making their digestive processes less efficient (Halitschke et al. 2001; Liang and McManus, 2002).

Insects' responses to plants' multiple signals

There is a growing consensus that signaling molecules interact in complex ways and can fine-tune plant defense reactions (Stotz et al. 2000; Glazebrook et al. 2003; Voelckel and Baldwin, 2004). When *N. attenuata* is attacked by *M. sexta* larvae, the expression of nearly 500 mRNAs is differently regulated (Hermsmeier, Schittko & Baldwin 2001). Similarly, in *Arabidopsis,* the regulation of nearly 700 mRNA genes belonging to insects as well as of pathogen-activated pathways has been observed (Schenk et al. 2000). Yet it is unlikely that all observed genes are involved in insect resistance. Perhaps a few genes encode direct defense products while some are involved in biosynthetic pathways that produce the defense compounds (Gatehouse, 2002). These same studies reported the down-regulation of genes involved in photosynthesis and primary metabolism, probably to allocate more resources to the production of defense compounds. This shift between growth and defense responses might alter the nutritional status of the plant as well, and influence insect growth and development. As a consequence of multidimensional changes in host-quality, solanaceous specialists like *M. sexta* may have developed coping strategies against many of the metabolites that plants elicit; such strategies may entail behavioral changes, such as altering either their food consumption rate or diet selection, or increasing their digestive efficiency, all of which increases the net allocation of energy from the ingested food to body mass.

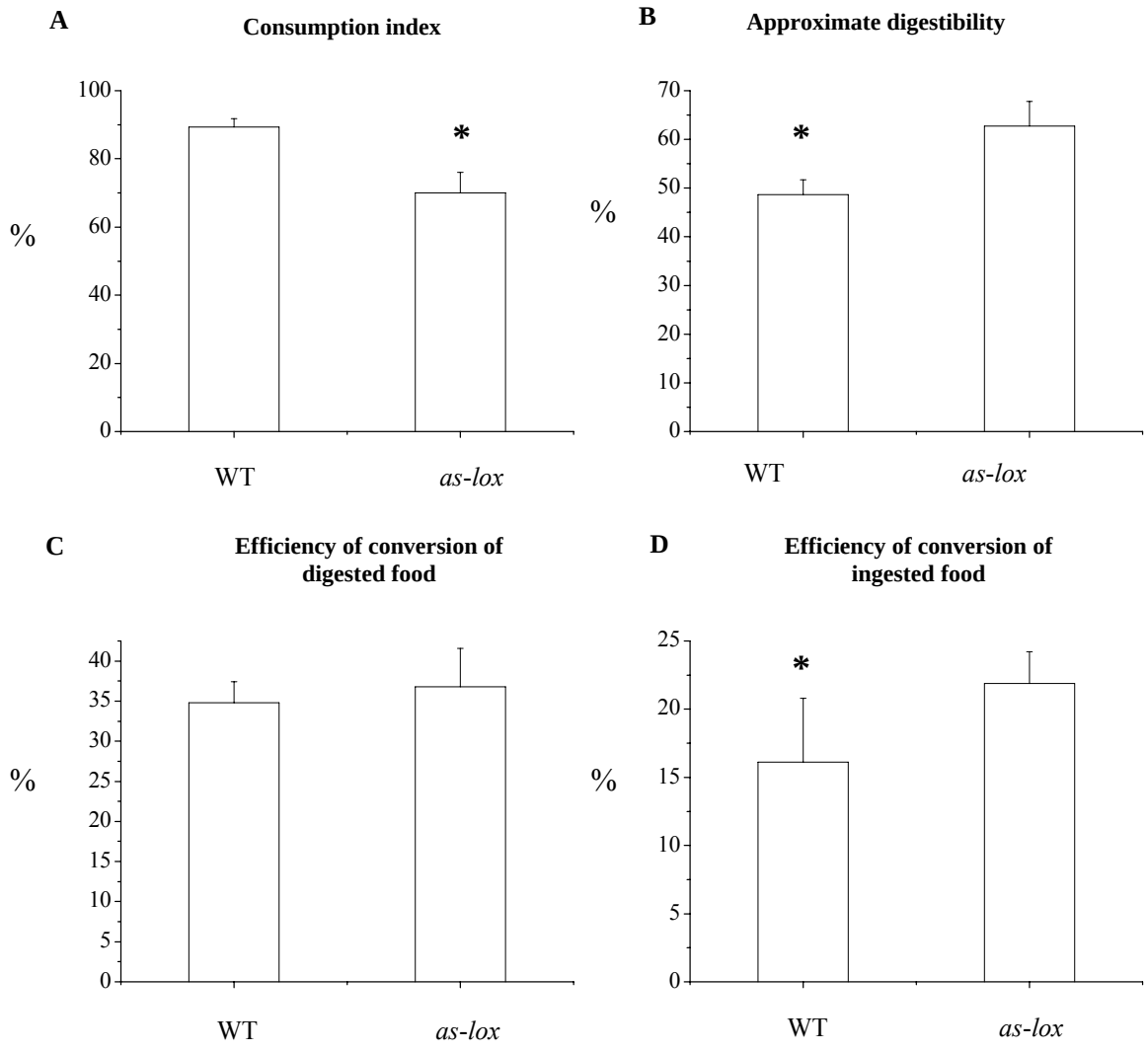

Figure 5. Mean (± SE) nutritional indices from larvae feeding on W+R treated *as-lox* transgenic and WT *Nicotiana attenuata* plants after 7 days of feeding. **A**) consumption index (CI), **B**) approximate digestion (AD), **C**) efficiency of conversion of digested food (ECD), and **D**) efficiency of conversion of ingested food (ECI). Significant changes in the CI, AD, and ECI can be observed among the derived nutritional variables. Asterisks indicate the level of significant differences between the two genotypes ($p < 0.05$).

Insects' nutritional requirements change as they move through their developmental stages and they need to ensure an adequate supply of nutrients from chemically defended plants.

Usefulness of nutritional indices in understanding plant defense

LOX3-dependent metabolites force larvae to reduce their food consumption (pre-ingestive) and ECD (post-ingestive). Surprisingly, in the 11-day trial, the reduction in ECD did not appear to be due to reduced AD. Two factors may be responsible: 1) it is known that ECD can vary considerably with the nutrient intake (Gordon 1959) and that insect attack results in a suppression of photosynthetic and primary metabolism genes in plants

(Hermsmeier et al. 2001; Hui et al. 2003), which may deprive larvae from ingesting essential macro-nutrients that would otherwise be used for increasing their ECD. For example, nitrogen fertilization has been observed to increase ECI and ECD in gypsy moths (Giertych et al. 2005); 2) differences in AD might be obscured in larvae that fed on *as-lox* ev as they progress into late instars. Increases in ECD in late instars might be a consequence of the increased AD in early instars. Because larvae in early instars cannot consume more food, the only way they can improve the efficiency of ingested food is by increasing the digestibility of the food they consume. The decreasing AD and increasing ECD observed in desert locusts (*Schislocerca gregaria*) as they moved from early to late instars were explained by shifts in food selection, digestive physiology, metabolic rates, and body composition (Lindroth, 1993).

We also observed that insects increase their CI in response to the reduced ECI, which in turn is due to the reduced ECD or AD. Insects confined to a low-quality plant commonly compensate for diminished quality by consuming more of a plant they might otherwise avoid (Price et al. 1980). Rather than benefiting the larvae, this compensation mechanism increases their exposure to defense compounds, which must subsequently be detoxified. This detoxification uses much of the derived energy that would otherwise be allotted to body mass, and as a result larval ECD decreases. To compensate for reduced body mass, larvae consume more.

Future directions

Our understanding of a plant's ability to resist attack from herbivores will be advanced when 1) all of the metabolites involved in insect resistance are identified; 2) genetic control over these metabolite pools is understood in sufficient detail to manipulate these pools *in planta*; and 3) studies similar to those described here are carried out in plants genetically modified for specific metabolite(s). Additionally, in order to understand how early responses in insects affect later responses, nutritional indices should be analyzed at different stages.

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Supplemental Materials and methods

Sources of errors and their control in Waldbauer assays

a) Variation in ECDs: ECD errors are strongly associated with errors in estimates of R, the only parameter not directly measured in a Waldbauer assay. Errors arise principally when insects have food in their guts at the time of weighing, which causes the amount of food eaten to be overestimated (Bowers, Stamp & Fajer 1991). We addressed this problem by separating larvae from their food for 4 h, collecting the frass produced during this period, and adding it to total frass pool.

b) Variation due to nutrient and water loss in leaves: Changes in tissue composition and loss of water from the excised leaf material contribute substantially to the errors in Waldbauer assays (Axelsson and Agren, 1979). Feeding trials were conducted at 75% RH to reduce moisture loss, and leaf material was changed every second day. Leaf material was supplied in one piece to avoid increased water loss from multiple wounds.

c) Variation due to excess food or dearth of food: As pointed out by Axelsson and Agren (1979) and van Loon (1991), the magnitude of errors arising from maintenance respiration of the food material increases with increasing food excess. In a pilot experiment, we determined the amount of food ingested per day and adjusted the mass of food given to reflect increases in consumption as larvae grew. To reduce the variation due to plant maintenance respiration, we selected leaf material from plants of the same age and nodal position, and excised them in the same way to standardize the amount of wounding.

d) Variation in the mass estimation: Considerable variation can occur during dry mass measures of insects, leaves, and frass. We dried material at 65^0 C for 3 days, and weighed them at least twice to ensure that a constant dry weight was attained. All samples were placed on a tray containing silica gel to avoid dried material absorbing moisture during weighing.

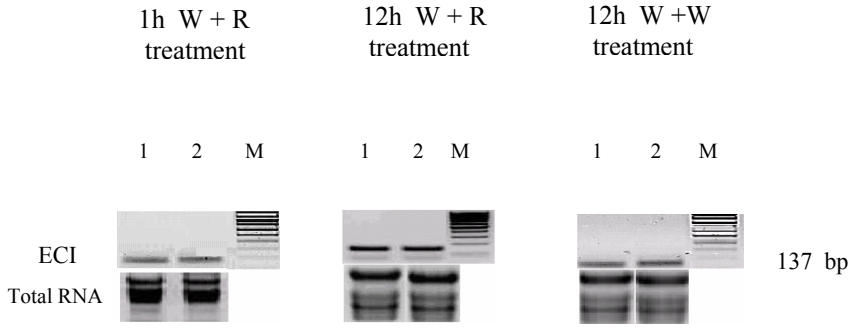

Figure S1. Loading controls for the LOX3, TPI, and PR-1 gene expression analysis presented in Fig. 1. A 137 bp, constitutively expressed ECI band amplified using 10ng cDNA from WT ev (1) and *as-lox* ev (2) treated either with W+R or W+W (1h and 12h) and the optimized total RNA used for cDNA synthesis served as loading controls. M represents the 100 bp gene marker.

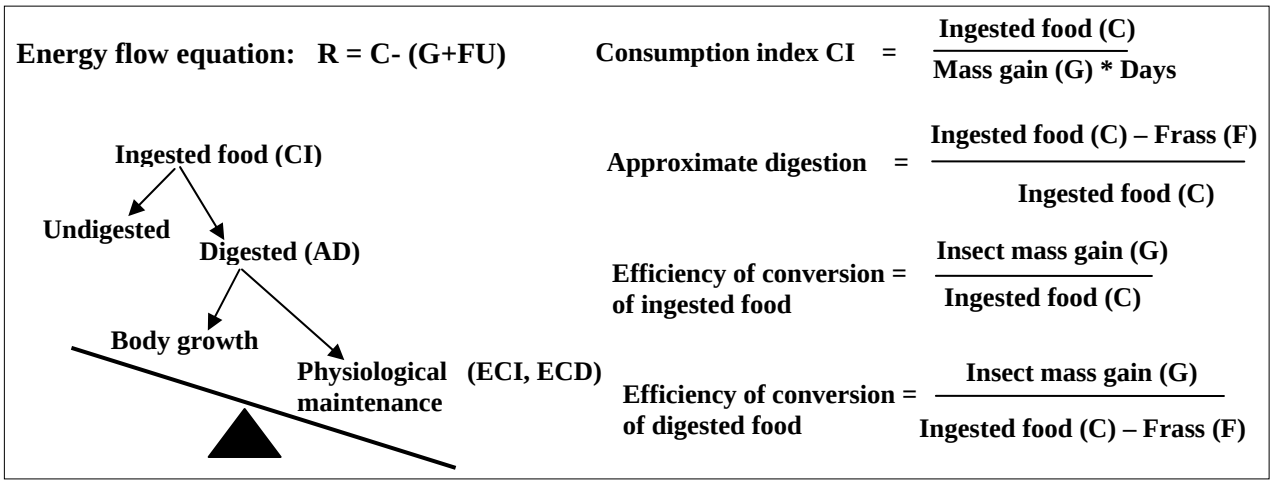

Figure S2. Schematic representation of biomass/energy flow in insects and the equations pertaining to the derivation of the Waldbauer nutritional indices. Body mass gain in insects is affected either during food consumption or digestion, or while allocating derived energy from the digested food to body mass. Different Waldbauer indices measure different components of insect nutrition (right). Growth and amount of food respired for physiological maintenance are inversely related (left). Decrease in growth without an increase in the total food consumed is attributed to increased respiration (R). Defense genes and their metabolites regulated by LOX3-dependent signaling exert an effect whereby larvae spend more energy derived from the digested food for physiological maintenance which compromises growth. C-amount of food consumed, G-gain in larval growth, and FU-amount of frass egested.

Supplemental Table1. Mean ± SE (standard error) primary and derived Waldbauer nutritional indices (primary and derived) from an 11-day assay. Wild type (WT) or *as-lox* plants were inoculated with *Agrobacterium*-harboring TRV constructs containing an empty vector (ev) and treated with W+R. CI is consumption index, AD is approximate digestibility, ECD is efficiency of conversion of digested food, and ECI is efficiency of conversion of ingested food.

Waldbauer nutritional indices	**WT ev**	***as-lox* ev**
Larval mass on plant (mg)	6.6 ± 1.1	15.83 ± 3.72
Larval mass excised leaf (mg)	7.9 ± 1.2	27.2 ± 4.55
Leaf consumed (mg)	51.3 ± 8.8	88.6 ± 10.05
Frass egested (mg)	23.8 ± 4.9	35.7 ± 4.94
CI (%)	83.9 ±12.5	37.5 ± 5.16
AD (%)	54.3 ± 7.2	59.4 ± 4.22
ECD (%)	27.4 ± 5.3	46.9 ± 4.98
ECI (%)	13.2 ± 2.6	27.3 ± 3.28

Manuscript 2

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Increased SA in *NPR1*–silenced plants antagonizes JA and JA-dependent direct and indirect defenses in herbivore-attacked *Nicotiana attenuata* in nature

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KEYWORDS: *Nicotiana attenuata*, jasmonic acid, salicylic acid, *NPR1*, *Spodoptera exigua*

Selected abbreviations:

OS	oral secretions
W	wounding
Na-*NPR1*	*Nicotiana attenuata* non-expressor of *PR-1*
JA	jasmonic acid
SA	salicylic acid
Na-*PR-1*	*Nicotiana attenuata* pathogenesis-related gene-1
SAR	systemic acquired resistance
VOC	volatile organic compounds
IR	induced resistance

Running title: NPR1 in induced resistance

Summary

The phytohormone jasmonic acid (JA) is known to mediate herbivore resistance, while salicylic acid (SA) and non-expressor of PR-1 (NPR1) mediate pathogen resistance in many plants. Herbivore attack on *Nicotiana attenuata* elicits increases in JA and JA-mediated defenses, but also increases SA levels and Na-*NPR1* transcripts from the plant's single genomic copy. SA treatment of WT plants increases Na-*NPR1* and Na-*PR1* transcripts. Plants silenced in *NPR1* accumulation by RNAi (ir-*npr1*) are highly susceptible to herbivore and pathogen attack when planted in their native habitat in Utah. They are also impaired in their ability to attract *Geocorus pallens* predators, due to their decreased ability to release *cis*-α-bergamotene, a JA-elicited volatile "alarm call." In the glasshouse, *Spodoptera exigua* larvae grew better on ir-*npr1* plants, which had low levels of JA, JA-isoleucine/leucine, *lipoxygenase-3* (*LOX3)* transcripts, and JA-elicited direct defense metabolites (nicotine, caffeoyl putrescine, and rutin) but high levels of SA and *isochorismate synthase* (*ICS*) transcripts, suggesting *de novo* biosynthesis of SA. A microarray analysis revealed the down-regulation of many JA-elicited genes and the up-regulation of SA biosynthetic genes. JA treatment restored nicotine and resistance to *S. exigua* in ir-*npr1* plants. We conclude that during herbivore attack, NPR1 negatively regulates SA production, allowing the unfettered elicitation of JA-mediated defenses; when *NPR1* is silenced, the elicited increases in SA production antagonize JA and JA-related defenses, making the plants susceptible to herbivores.

Introduction

Plants employ inducible defenses to prevent their tissues from being damaged by herbivores and pathogens. Defenses elicited in response to insect and pathogen attack are known as Induced Resistance (IR) and Systemic Acquired Resistance (SAR), respectively. IR and SAR are associated with the elicitation of a distinct set of defense genes; these genes are largely mediated by the phytohormones JA and SA (Ward *et al.,* 1991; Kessler and Baldwin, 2002; Delaney *et al.,* 1995; Cheong *et al.,* 2002). It has become apparent that herbivore or pathogen attack frequently recruits not one but many signal cascades. For example, bacteria (*Pseudomonas syringae*) can activate both the SA and the JA pathways in *Solanum esculentum* (Stout, Fidantsef et al. 1999), while in *Arabidopsis*, herbivore (*Pieris rapae*) damage elicits both JA- and SA-dependent defenses (De Vos *et al.,* 2006). The specificity of responses in defense gene expression to particular attackers seems to be the result of a network of interconnecting signal cascades that cross-communicate (Feys and Parker, 2000; Glazebrook, 2001; Thomma *et al.,* 2001, Heidel and Baldwin, 2004). Cross-communication among different signaling cascades provides plants with the regulatory potential that is needed to tailor their responses to the diverse herbivore species that attack them (Walling, 2000). Although cross-communication among different signaling cascades in plants infected by

pathogens has been extensively studied, not much is known about cross-communication in plants attacked by different types of herbivores. Studies have reported the elicitation of SA and its marker genes (e.g., *NPR1, PR-1*) after herbivore attack (Glazebrook, 2001; Heidel and Baldwin, 2004), yet their exact role in IR remains unknown.

The non-expressor of *PR-1* (NPR1) is known to be a major molecular player in SAR. NPR1 functions as a transducer of SA, which is produced after pathogen attack. NPR1 was first identified in *Arabidopsis* in genetic screens for SAR-compromised mutants (Cao *et al.,* 1994; Delaney *et al.,* 1995). Pathogen attack results in changes in cytosolic cellular redox as well as increases in the levels of SA; these increases cause the constitutively present NPR1 protein to de-polymerize and form monomers (Mou, Fan et al. 2003) which migrate to the nucleus where they associate with transcription factors (TGA family) that induce pathogenesis-related (*PR)* defense genes (Zhang *et al.,* 1999). However, NPR1's function is not restricted to SA-dependent responses; it also interacts with different signaling cascades in response to different attackers. For example, during induced systemic resistance (ISR: a biologically elicited, systemic defense response activated when roots are colonized by particular strains of non-pathogenic rhizobacteria), JA and ethylene responses are mediated via NPR1, independently of SA (Pieterse and Loon, 2004). Among other functions, NPR1 can negatively regulate SA biosynthesis during pathogen attack (Shah 2003). Recently, NPR1 has been shown to mediate the SA-induced suppression of JA-dependent responses (Spoel, Koornneef et al. 2003). These studies have highlighted the diverse roles that NPR1 plays in plants. The *NPR1* gene encodes a protein with a BTB/BOZ domain and an ankyrin-repeat domain; both domains are characteristic of proteins with diverse functions (Bork, 1993; Cao *et al.,* 1997; Aravind and Koonin, 1999).

Several studies with *Arabidopsis* have suggested different causal associations between SA and NPR1 expression and herbivore/pathogen performance. For example, *Trichoplusia ni* larvae fed more on constitutive SAR mutants with elevated SA levels than on wild-type (WT) plants, but eliciting SAR with avirulent bacteria, a process which is typically accompanied by elevated SA levels, decreased insect feeding (Cui *et al.,* 2002). In another study, *Spodoptera littoralis* larvae fed less on SAR-compromised *npr1* mutants than on WT plants; SA treatment enhanced feeding only in *npr1* mutants in which SA does not induce SAR (Stotz *et al.,* 2002). These studies suggest that SA and NPR1 have the potential to inhibit JA responses, but the mechanism remains unclear. Moreover, most of these studies have been performed with *Arabidopsis* grown under laboratory conditions with herbivores or pathogen

strains whose ecological relevance is not known. An understanding of NPR1's role in mediating herbivore resistance in plants growing in their natural habitats would be valuable.

IR is well studied in *Nicotiana attenuata*, a native plant of the southwestern United States. *N. attenuata* is an annual plant that grows in post-fire environments; as a result, it has to re-establish itself regularly with new plant populations and unpredictable herbivore communities. Because it grows in the post-fire niche, *N. attenuata* has adapted to be able to respond to attack from different herbivores using different blends of secondary metabolites (Kessler and Baldwin, 2002). How *N. attenuata* generally responds to herbivory has been studied using the specialist herbivore *Manduca sexta*. Allowing *M. sexta* to feed on *N. attenuata* or applying larval oral secretions (OS) to puncture wounds in leaves elicits a JA burst, which in turn mediates the accumulation of various direct defense metabolites (Halitschke and Baldwin, 2004). Some of the main anti-herbivory metabolites studied are nicotine (Baldwin, 1999), caffeoyl putrescine, rutin, and diterpene glycoside (Keinanen *et al.,* 2001), as well as anti-digestive trypsin protease inhibitors (TPIs) (van Dam *et al.,* 2001; Zavala *et al.,* 2004). The OS-elicited JA burst also influences the accumulation of several indirect defense metabolites. Among the indirect defense compounds *N. attenuata* produces are the volatile organic compounds (VOCs), which attract predators of *M. sexta* eggs and larvae (Halitschke *et al.,* 2000; Kessler and Baldwin, 2001). Plants which are genetically engineered to accumulate less JA (Halitschke and Baldwin, 2003; Kessler *et al*., 2004) or are unable to respond to JA (Paschold *et al.,* 2007) have demonstrated that JA acts as a major transducer of signals that are essential to plant defense.

Here we address three questions about NPR1's role in *N. attenuata*'s interactions with its native herbivores: Does NPR1 mediate cross-communication between SA and JA pathways to optimize the function of its direct and indirect defenses? If so, how does this optimization come about? And how does NPR1 expression influence JA-mediated direct and indirect defenses in plants grown under natural conditions? We transformed *N. attenuata* plants with an RNAi construct harboring a fragment of Na-*NPR1* in an inverted-repeat orientation (ir-*npr1*) to silence expression of the endogenous Na-*NPR1* gene. We compared the performance of larvae of the second most important native lepidopteran herbivore of *N. attenuata*, *Spodoptera exigua,* on ir-*npr1* and WT lines in experiments conducted in the glasshouse as well on native herbivores in the plant's native habitat, the Great Basin Desert. To understand the resistance phenotypes, we measured the production of different phytohormones (total SA, free SA, conjugated SA, JA, JA-amino acid conjugates), direct defense metabolites (nicotine, rutin, and caffeoyl putrescine), indirect defense metabolites

(VOCs), and gene expression profiles. The results demonstrate that Na-NPR1 and its associated phytohormone, SA, influence JA-dependent IR and in doing so influence both direct and indirect defenses.

Results

Isolation and characterization of *NPR1* in *N. attenuata* (Na-*NPR1*)

Using the sequence information of *Nicotiana tabacum NPR1* (Liu *et al.,* 2002) (NCBI accession number-AF480488), primers were designed to isolate a 335 bp non-intronic partial cDNA fragment and a full-length ORF from W+OS (*M. sexta*)-elicited leaves of WT *N. attenuata* (sequence submitted in NCBI database-accession number-DQ351939) (Fig. S1). *N. tabacum* (*NPR1)* and *N. attenuata (NPR1)* are almost identical (> 97%) at the nucleotide level. In order to determine the copy number of *NPR1* in *N. attenuata*, a Southern hybridization using the 335 bp partial fragment as the probe was carried out. Analysis revealed that *NPR1* is a single-copy gene in *N. attenuata*'s genome (Fig. S2). Silencing Na-*NPR1* expression by an *Agrobacterium*-mediated transformation procedure (Krugel, Lim et al. 2002) using a pRESC5 transformation vector containing an inverted-repeat construct of Na-*NPR1* (Fig. S3) (see experimental procedures) yielded three independently transformed lines (Fig. S4A). Levels of Na-*NPR1* transcripts after SA treatment were significantly decreased (at least 50%) in all three ir-*npr1* lines compared to levels in identically treated WT plants (Fig. S4B; ANOVA $F_{3, 12} = 5.460$, $P < 0.001$). Two ir-*npr1* lines (174 and 213) were selected for all further experiments. In *Arabidopsis* and tobacco, *NPR1* is known to be SA responsive, and pathogen attack or SA treatment activates *NPR1*. Activated NPR1 in turn influences the accumulation of transcripts of pathogenesis-related genes, the best studied of which is *PR-1* (Ward *et al.,* 1991). In order to determine if *N. attenuata's NPR1* (Na-*NPR1*) responds similarly, we analyzed the transcript accumulation of Na-*NPR1* and Na-*PR1* using quantitative real-time PCR (qRT-PCR). After SA treatment, WT *N. attenuata* plants accumulated higher levels (> 1-fold) of Na-*NPR1* transcripts (Fig. 1; ANOVA $F_{5, 12} = 7.04$, $P = 0.002$) as well as Na-*PR-1* transcripts (> 1-fold) (Fig. 1; ANOVA $F_{5, 12} = 25.36$, $P < 0.0001$) compared to similarly treated ir-*npr1* plants. Control plants treated only with water on the other hand, revealed basal levels of Na-*NPR1* but not Na-*PR-1* in all genotypes. In summary, silencing Na-*NPR1* reduced Na-*PR1* accumulation, suggesting that Na-*NPR1* regulates Na-*PR-1* expression in a SA-dependent manner. These results confirmed that Na-*NPR1* is indeed

responsive to SA and suggested that Na-*NPR1* is also involved in regulating the expression of *PR* genes, which in cultivated tobacco are correlated with disease resistance.

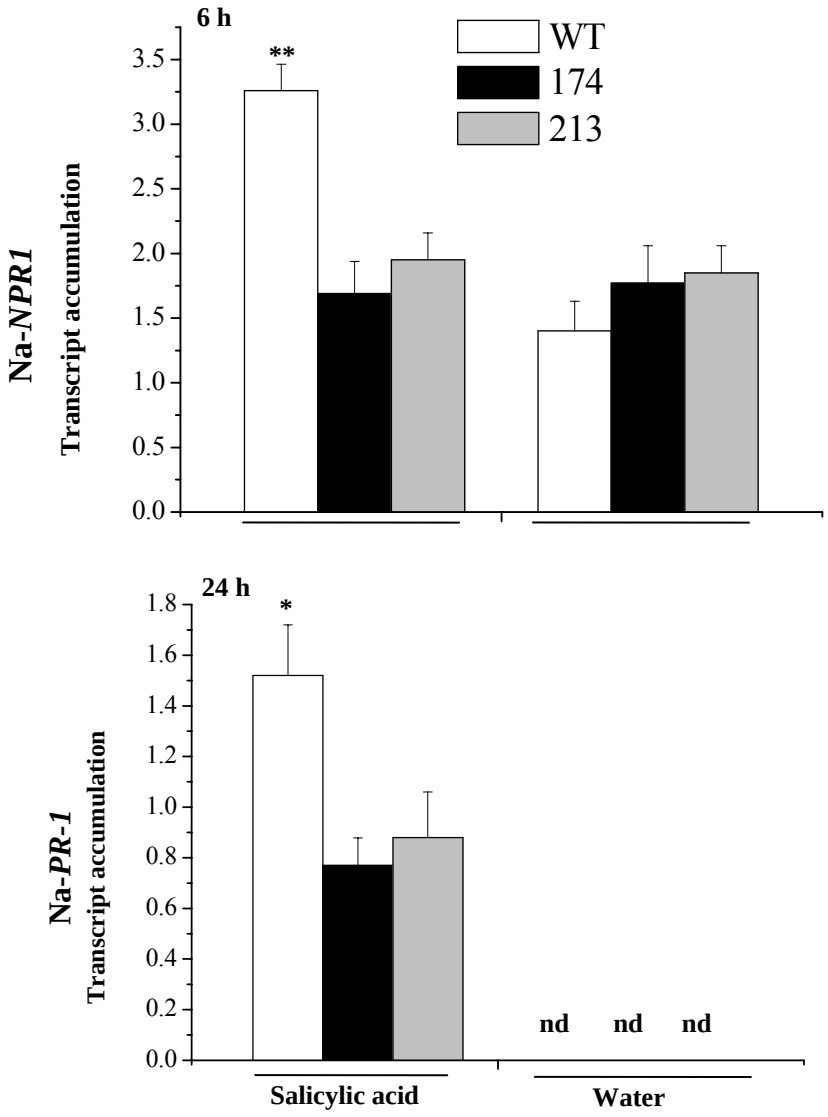


Figure 1. Na-*NPR1* and Na-*PR-1* transcript analysis. Quantitative real-time PCR (qRT-PCR) analysis of Na-*NPR1* (6 h) and Na-*PR-1*(24 h) transcript accumulation in WT plants and ir-*npr1* lines (174 and 213) of *N. attenuata* in response to spraying with 1 mM SA or water. 100 ng of cDNA from 3 replicate plants of SA- and water-treated leaves was used in the analysis. A constitutively unregulated sulfite reductase gene (*ECI*) was used for normalization. Asterisks indicate significant differences among WT plants and ir-*npr1* lines (174 and 213) for the respective treatments ($P < 0.05$ (*), $P < 0.01$ (**) (N=3) (nd =not detected).

Characterizing Na-*NPR1* gene expression and protein accumulation in response to herbivore attack

We measured the kinetics of transcript accumulation in WT and ir-*npr1* plants in response to *S. exigua* attack. The qRT-PCR results showed that Na-*NPR1* transcript accumulation was significantly higher in WT compared to ir-*npr1* plants 1 h (Fig. 2A; ANOVA $F_{5, 12} = 5.309$, $P = 0.008$) and 6 h (Fig. 2A; ANOVA $F_{5, 12} = 25.8$, $P < 0.001$) after the start of *S. exigua* attack. In addition, we also measured Na-NPR1 protein accumulation 24 h after the start of herbivore attack. Western blot analysis revealed that Na-NPR1 was present constitutively. Moreover, *S. exigua* damage marginally increased protein levels in WT plants compared to undamaged WT plants after 24 h. Finally, Na-NPR1 accumulation in *ir-npr1* plants compared to WT plants was substantially reduced (Fig. 2B).

Na-*NPR1*-silenced plants are susceptible to herbivores and pathogens in nature

To determine if Na-NPR1 mediates responses influencing the resistance of *N. attenuata* to herbivores and pathogens under natural conditions, we transplanted size-matched WT and ir-*npr1* (213) pairs into their native habitat in the Great Basin Desert (near Santa Clara, UT, USA) and compared the extent of damage on both genotypes. Total herbivore damage was significantly higher on ir-*npr1* plants (88% on day 25 after transplanting) than on WT plants during a 5-day observation period (Fig. 3A; ANOVA $F_{5, 60} = 3.381$, $P = 0.009$). Among the individual herbivores, grasshoppers inflicted significantly more damage (1.3-fold on day 25) on ir-*npr1* (213) than on WT plants (Fig. 3B; ANOVA $F_{5, 60} = 3.86$, $P = 0.004$). However, flea beetles (*Ephitrix sps*) did not damage ir-*npr1* (213) plants significantly more than WT plants (Fig. 3C; ANOVA $F_{5, 60} = 0.609$, $P = 0.693$). Interestingly, ir-*npr1* (213) plants also showed significantly more (2-fold on day 25) symptoms that resembled bacterial spots than did the WT plants (Inset: Fig. 3D; ANOVA $F_{5, 60} = 2.48$, $P = 0.01$). The disease symptoms appeared two days after a brief rain, which may have vectored soil bacteria to the leaf surfaces via raindrop splash. Because all plants were in the rosette stage - namely, leaves were in direct contact with the ground - they were lightly covered in soil after the rain. We subsequently identified two *Pseudomonas sps* (an unidentified species of *Pseudomonas*-strain 4 and *Pseudomonas jessenii*) from the infected field samples (Table S1). Both strains of *Pseudomonas* were tested on WT *N. attenuata* under glasshouse conditions and only *Pseudomonas sps*-strain 4 was found to be pathogenic (Fig. S5). In summary, we found that Na-*NPR1*-silenced plants were more susceptible than WT plants to herbivores and pathogens in nature.

Na-*NPR1* silencing inhibits JA-mediated indirect defenses in nature

In nature, *N. attenuata*'s JA-dependent responses are known to elicit the release of volatile organic compounds (VOCs), which in turn attract *Geocorus pallens* (a lepidopteran egg and larval predator) to herbivore-attacked or oral secretion (OS)-elicited plants (Kessler and Baldwin, 2001).

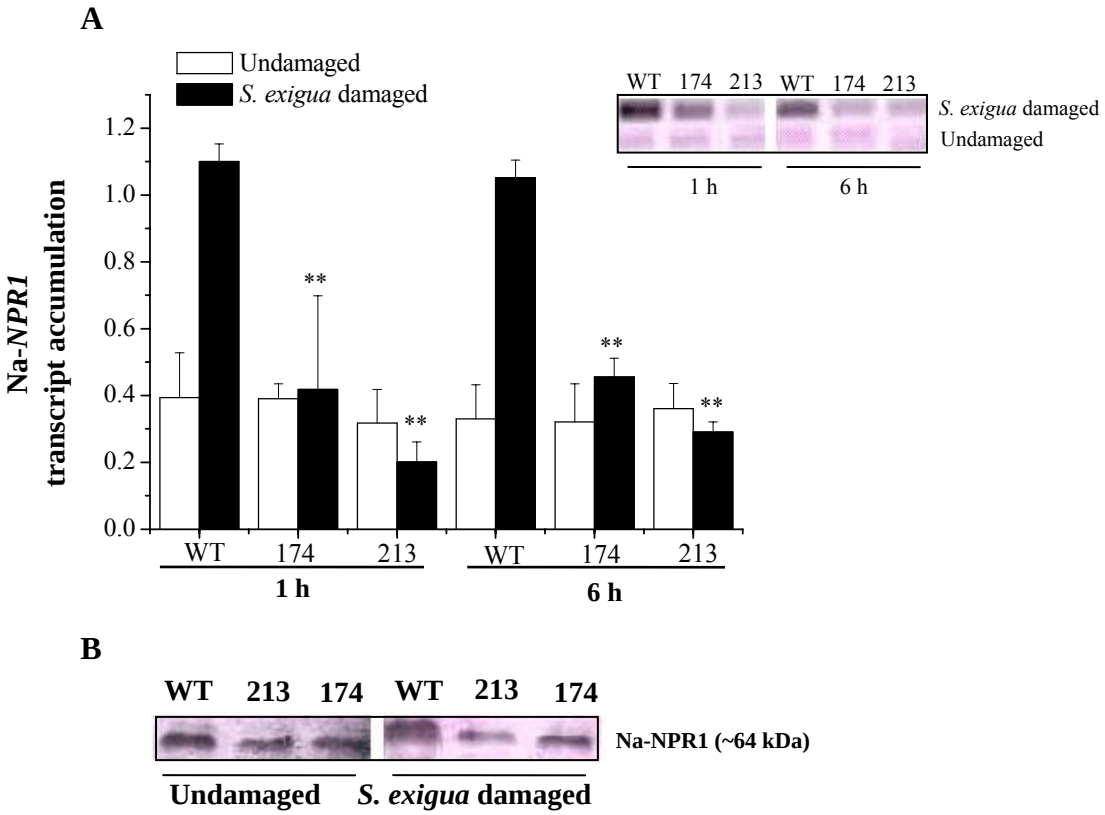


Figure 2 A) Na-*NPR1* transcript accumulation in WT plants and ir-*npr1* lines (174 and 213) of *N. attenuata* in response to *S. exigua* attack. The transcripts were analyzed by quantitative real-time PCR (qRT-PCR) and expressed as the mean (± SE) of 3 replicate plants in arbitrary units from 100 ng cDNA prepared from RNA samples extracted 1 and 6 h after continuous feeding by *S. exigua* larvae. A constitutively unregulated sulfite reductase gene (*ECI*) was used for normalization. Asterisks indicate significant differences among *S. exigua*-damaged WT plants and ir-*npr1* lines (174 and 213) at the respective harvest times. $P < 0.01$ (**) (N=3). **Inset**: the real-time PCR products from the same experiments separated on a 1.7% agarose gel. **B)** Western blot analysis of expression of Na-NPR1 protein in WT plants and ir-*npr1* lines (174 and 213). 20 µg of total leaf protein was separated on an 8.0% SDS-polyacraylamide gel and visualized by immunoblotting with Na-NPR1 polyclonal antibody (for loading control see Figure. S11).

To determine if this indirect defense is altered in an ir-*npr1* line (213), we analyzed the ability of WT and ir-*npr1* (213) plants to attract predators after OS elicitation with an egg predation

assay that has been developed to measure a plant's ability to attract *G. pallens* to *M. sexta* eggs (Kessler and Baldwin, 2001). *M. sexta* eggs and larvae are a natural food for this abundant predator, and since gluing eggs to a plant does not elicit a VOC response, this assay allows predation rates to be measured before and after OS-elicited VOC releases (Kessler and Baldwin, 2001).

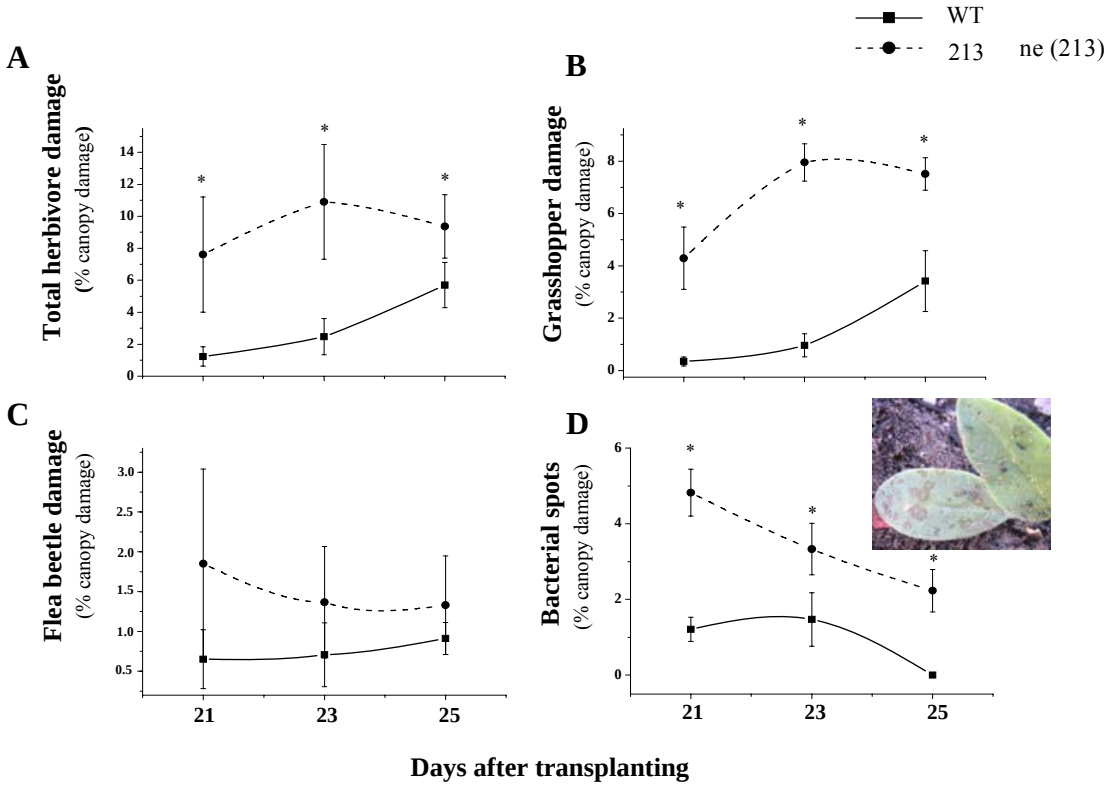


Figure 3. Herbivory on pairs of size-matched ir-*npr1* (213) and WT *N. attenuata* plants growing in *N. attenuata*'s native habitat in the Great Basin Desert. Damage caused by **A**) all herbivores, **B**) grasshoppers, **C**) flea-beetles (*Epitrix hirtipennis*), and **D**) bacteria *Pseudomonas spps* (**inset:** Bacterial damage symptoms). Damage was measured as % of total canopy area damaged 21, 23, and 25 days after plants were transplanted into the field. The asterisk (*) indicates a significant difference between WT and 213 plant pairs at $P < 0.05$ (N=15 pairs).

G. pallens predation on eggs results in a characteristic small feeding hole through which the contents of the egg are emptied. Such emptied eggs are transparent and papery and readily distinguished from non-predated eggs, which appear greenish (Inset: Fig. 4A). For 42 h after eggs were glued to leaves and before plants were elicited with *M. sexta* OS, few eggs were predated and there were no significant differences in the number of eggs predated

between WT and ir-*npr1* (213) lines. However, within 17 h of OS elicitation, 41% of the 35 eggs (5 eggs each on 7 replicate plants) affixed to WT plants had been attacked compared to only 16% of the 35 eggs glued to ir-*npr1* line 213 (Fig. 4A; ANOVA $F_{9, 57} = 6.708$, $P < 0.001$). Similar differences were observed after 25 h. Consistent with the predation rate data, after elicitation WT plants attracted more than three times the number of *G. pallens* compared to ir-*npr1* plants (213) (Fig. 4B; ANOVA $F_{9, 57} = 3.39$, $P = 0.02$).

G. pallens adults and larvae are known to be attracted to herbivore-attacked plants by OS-elicited VOCs which the plants release into their surroundings. To determine why ir-*npr1* plants were less able to attract predators, we trapped the headspace VOCs of field-grown plants before and after OS elicitation. We analyzed the VOCs and the GLVs from ir-*npr1* (213) and WT plants. The release of terpenoid VOC *cis*-α-bergamotene in particular was significantly lower (51%) from ir-*npr1* than from WT plants (Fig. 4C; ANOVA, $F_{1,12} = 8.93$, $P = 0.013$). This finding is consistent with previous field work with *N. attenuata* which highlights the role of *cis*-α-bergamotene in attracting *G. pallens* (Kessler and Baldwin, 2001). Interestingly, other VOCs, such as β-pinene, germacrene, limonene, and *cis*-jasmonol, did not differ significantly between WT plants and ir-*npr1* line 213 (Table S2), suggesting that these compounds are independent of the Na-NPR1-mediated response. In addition, the level of GLVs such as (z)-3-hexanol and (z)-3-hexanol acetate, which require a functional Na-HPL (hydroperoxy lyase), released from WT plants and ir-*npr1* line 213 did not differ (Table S2).

Na-*NPR1* silencing increases susceptibility to *S. exigua* and decreases direct defenses

Under field conditions, we observed that ir-*npr1* plants (line 213) were more susceptible than WT plants to generalist grasshoppers. Moreover, in an initial planting early in the growing season, ir-*npr1* plants were heavily attacked by *S. exigua* larvae. Although these larvae were not found in subsequent plantings, these results prompted us to measure *S. exigua's* performance in the glasshouse. We conducted two experiments. For the first, we used *S. exigua* larvae that had been reared for 3 days on WT *N. attenuata* plants. Larvae that fed on ir-*npr1* lines (174 and 213) gained significantly more mass (49%) by the end of day 9 than those that fed on WT plants (Fig. 5A; ANOVA, $F_{2, 24} = 2.625$, $P = 0.03$).

In the second experiment, we used larvae that had been reared on artificial diet instead of WT *N. attenuata* plants. In this experiment, the difference in performance of larvae that fed on ir-*npr1* lines (174 and 213) compared to WT plants was much larger than the difference in performance observed in the first experiment. When weighed on day 6 of the experiment,

larvae that fed on ir-*npr1* lines (174 and 213) were > 1.0-fold times larger than those that fed on WT plants (Fig. 5B; ANOVA, $F_{2, 24} = 7.525$, $P = 0.001$).

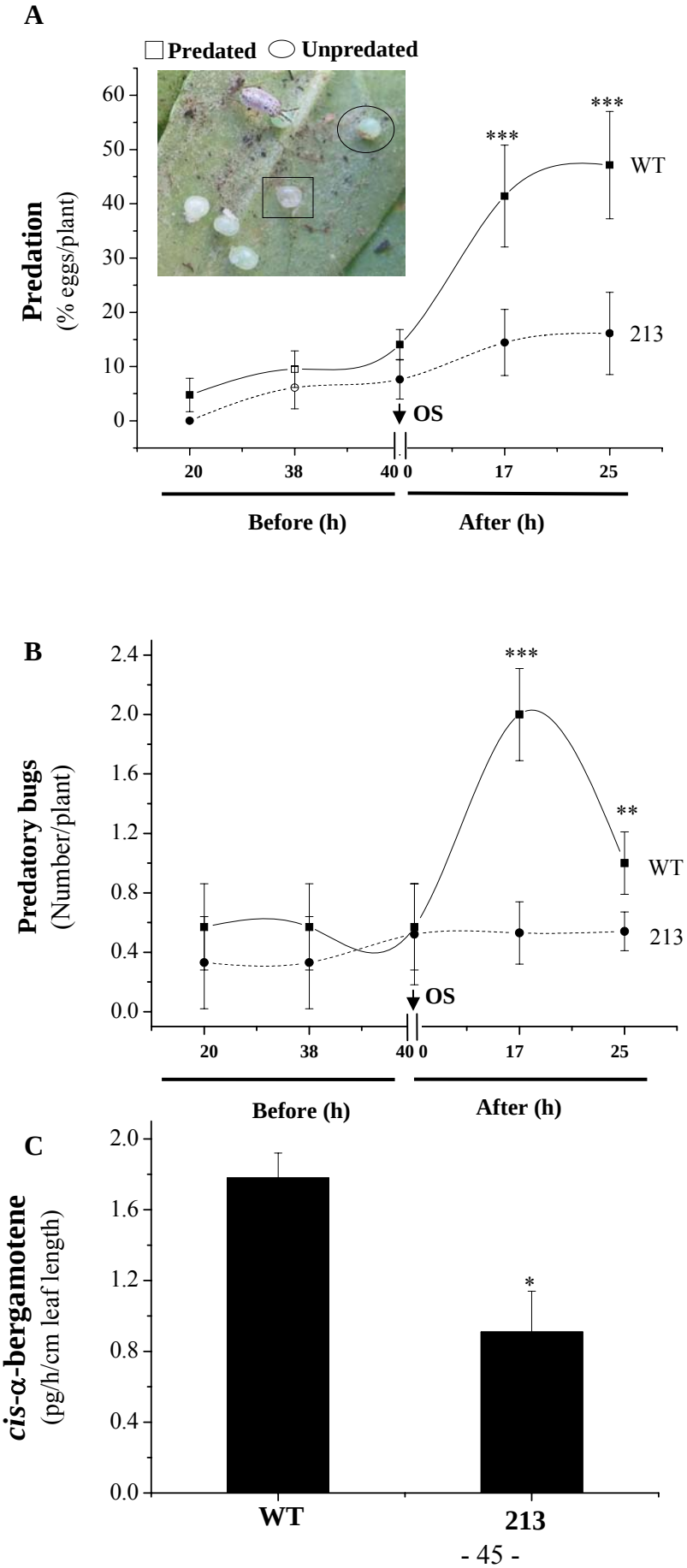


Figure 4. Effect of Na-*NPR1* on indirect defenses in nature. **A)** Mean (± SE) percentage of *M. sexta* eggs predated per plant on WT (solid line) and ir-*npr1* (line 213; dashed line) plants before and after elicitation using *M. sexta* OS (N=7 plants pairs). Five eggs were glued on the second stem leaf of each plant; predation rates from *Geocoris pallens* were monitored for 42 h before OS elicitation and for an additional 25 h after OS elicitation. **Inset:** Picture shows *G. pallens* predating *M. sexta* eggs. **B)** Mean (± SE) number *G. pallens* found on WT (solid line) and ir-*npr1* (line 213; dashed line) plants before and after OS (*M. sexta*) elicitation (N=7 plants pairs). **C)** Mean (± SE) emission of *cis*-α-bergamotene from WT and ir-*npr1* plants (line 213) 12 h after a second treatment with OS. Asterisk indicates significant differences between ir-*npr1* and WT plants (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, N=7).

The results from these two experiments demonstrate that silencing Na-*NPR1* increases the performance of *S. exigua* larvae, and suggest that prior exposure to a WT *N. attenuata* diet allows the larvae to better cope with *N. attenuata*'s defenses.

To determine if SA and JA treatments could help explain the reduced resistance to *S. exigua* in the ir-*npr1* lines, JA and SA (each 1mM), and water were sprayed on separate plants of WT and ir-*npr1* lines (174 and 213) that had been fed on by *S. exigua* for 3 days beforehand. The reason for using *S. exigua*-damaged plants was to make sure that Na-NPR1 and its dependent responses were activated in all of the plants prior to the treatments. When larval mass was measured on the 9th day, we found the following: 1)Larvae that fed on JA-treated WT plants and on ir-*npr1* lines (174 and 213) increased their body mass up to 85% (day 0 to day 9), while those feeding on SA-treated WT plants and on ir-*npr1* lines (174 and 213) were nearly 2.4-fold larger on day 9 than on day 0 (Fig. 5C; ANOVA, $F_{17, 145} = 13.66$, $P < 0.001$). 2) Treatment with JA or SA abolished the differences in larval performance among the lines (Fig. 5C). Larvae that fed on SA-treated WT plants and ir-*npr1* lines (174 and 213) gained similar amounts as those fed on water-treated ir-*npr1* lines (174 and 213), but the mass of all these larvae was significantly higher (2.2-fold) than the mass of those that fed on water-treated plants (Fig. 5C). In brief, these results demonstrate that SA and JA treatments have opposing effects on larval mass gain; mass gain is not influenced by the genotype; and a controlled production of SA in WT plants seems vital for resisting *S. exigua*.

Nicotine is known to be an effective defense against generalist herbivores including *S. exigua* larvae (Steppuhn *et al.,* 2004). Interestingly, after 72 h of feeding by *S. exigua*, nicotine levels in ir-*npr1* lines (174 and 213) were at least 62% lower than in WT plants (Fig. 6A; ANOVA, genotype: $F_{17, 52} = 8.539$, $P < 0.0001$).

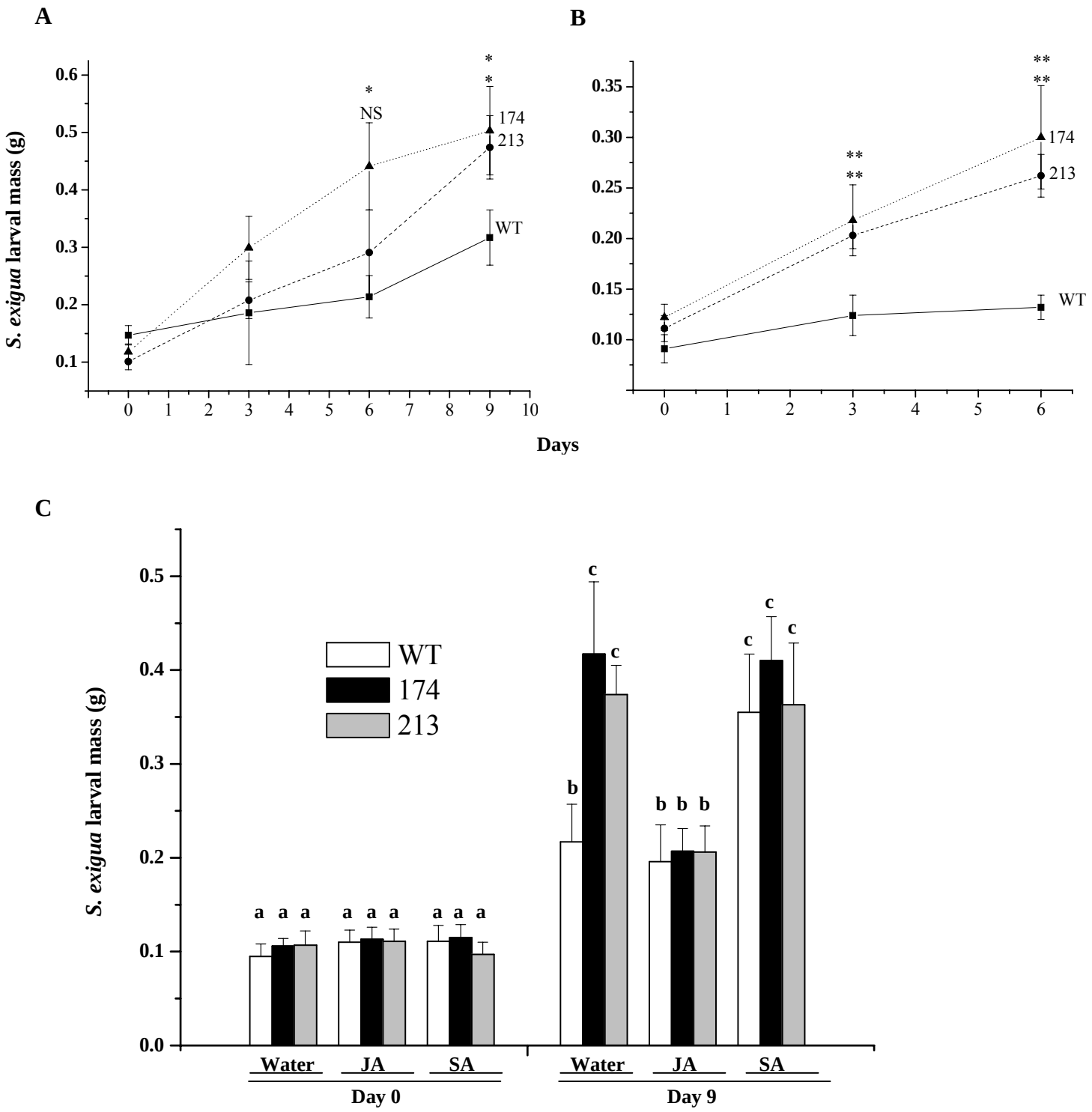


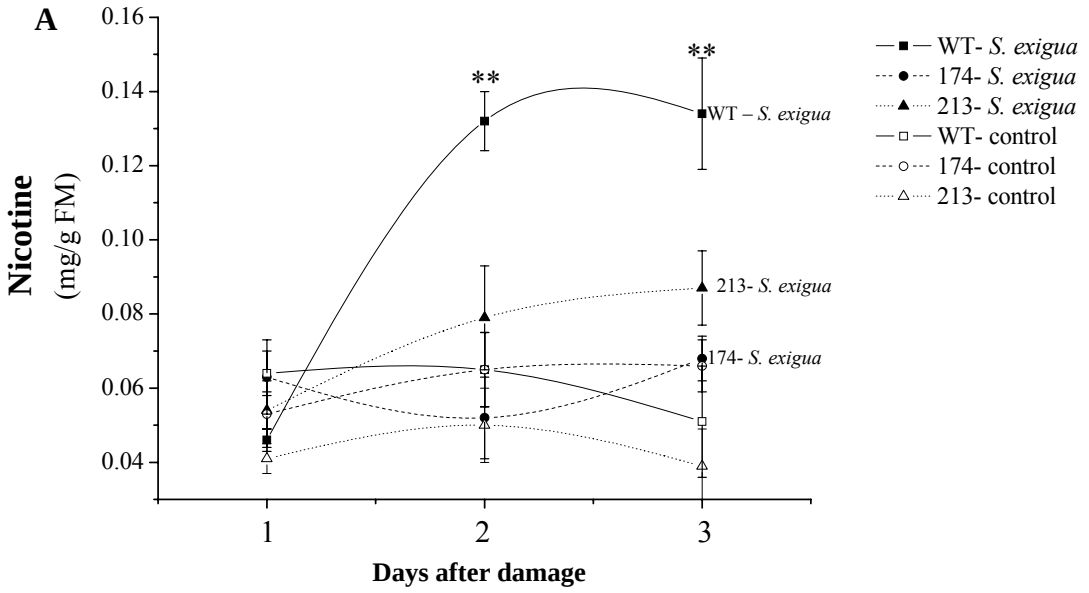
Figure 5. Silencing Na-*NPR1* increases susceptibility of *N. attenuata* to attack from *S. exigua* larvae. **A)** Mean (± SE) mass gain of *S. exigua* larvae on WT plants and ir-*npr1* lines 174 and 213. First-instar larvae were reared on WT *N. attenuata* for three days before being placed on WT and ir-*npr1* lines. **B)** Mean (± SE) mass gain of *S. exigua* larvae on WT plants and ir-*npr1* lines 174 and 213. First-instar larvae were reared on artificial diet for a week before being placed on the plants. In both experiments, larvae performed better on ir-*npr1* lines. Asterisks indicate significant differences among larvae feeding on WT and ir-*npr1* plants (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, N=15). **C)** Mean (± SE) mass of *S. exigua* larvae feeding on water, JA- and SA-treated WT plants, and ir-*npr1* lines (174 and 213). Before the treatments, WT plants and ir-*npr1* lines (174 and 213) were

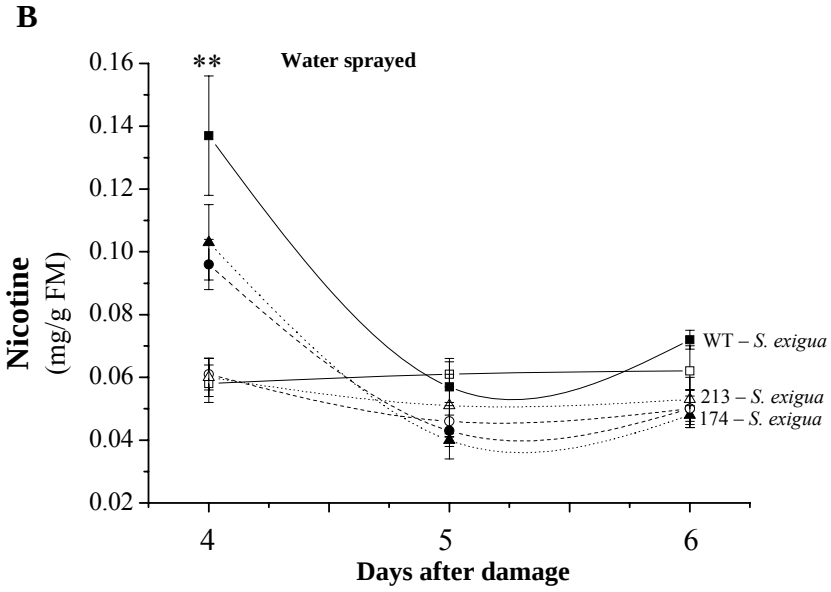
fed upon by *S. exigua* for 3 days to activate Na-NPR1 and its dependent responses. At the beginning of the 4th day, larvae were removed and plants were treated with water, JA, and SA. At the end of 4th day fresh larvae of uniform mass (day 0) were placed on water, and JA- and SA-treated WT plants, and ir-*npr1* lines (174 and 213), and their final mass was recorded after 9 days. Different letters indicate significant differences between treatments and genotypes (N=15).

We also analyzed the concentrations of two other potential defense metabolites: caffeoyl putrescine, which like nicotine is elicited by JA signaling, and rutin, which is not (Keinanen, Oldham et al. 2001). Caffeoyl putrescine, which was observed only in induced tissues, occurred at concentrations > 2.0-fold lower in ir-*npr1* lines (174 and 213) than in WT lines (Fig. S6; ANOVA, genotype: $F_{17, 52} = 9.814$, $P < 0.0001$). Similarly, levels of rutin were at least 60% lower in ir-*npr1* lines (174 and 213) than in WT lines (Fig. S6; ANOVA, genotype: $F_{17, 52} = 6.44$, $P < 0.0001$). These results demonstrate that Na-NPR1 expression influences both JA-dependent and JA-independent defense metabolites.

JA treatment restores nicotine levels in *NPR1*-silenced *N. attenuata* plants

Nicotine accumulation in *N. attenuata* is known to be JA-dependent, and treating JA-deficient *LOX3*-silenced plants with JA restores nicotine to levels similar to those found in WT plants (Halitschke and Baldwin, 2003). Since *S. exigua*-damaged ir-*npr1* plants accumulate less nicotine than do WT plants, we asked if JA treatment could complement the nicotine deficits in ir-*npr1* plants. We used ir-*npr1* and WT *N. attenuata* plants that had been attacked for 3 days by *S. exigua* larvae to ensure that the plants were activated for Na-NPR1 and its dependent responses. Twenty-four hours after treatment, a moderate increase in nicotine levels (at least 40%) was observed in WT and ir-*npr1* plants sprayed with JA compared to those sprayed with water (control plants). In both treatments (water and JA) ir-*npr1* plants accumulated significantly less nicotine than did the WT plants (Fig. 6B and C; ANOVA, genotype: $F_{1, 4} = 16.47$, $P = 0.015$), which is not surprising given that nicotine concentrations reflect a plant's life-time nicotine production (Baldwin and Ohnmeiss, 1994; Ohnmeiss and Baldwin, 1994). However, 48 and 72 h after JA treatment, WT and ir-*npr1* plants accumulated similar and significantly higher (1.9-fold) nicotine levels compared to WT and ir-*npr1* plants sprayed with water (Fig. 6 B and C; ANOVA, genotype: $F_{17, 36} = 53.85$, $P < 0.001$).


A
Nicotine
(mg/g FM)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
1
2
3
Days after damage
**
**
WT – S. exigua
213- S. exigua
174- S. exigua
WT- S. exigua
174- S. exigua
213- S. exigua
WT- control
174- control
213- control


B
Water sprayed
Nicotine
(mg/g FM)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
4
5
6
Days after damage
**
WT – S. exigua
213 – S. exigua
174 – S. exigua

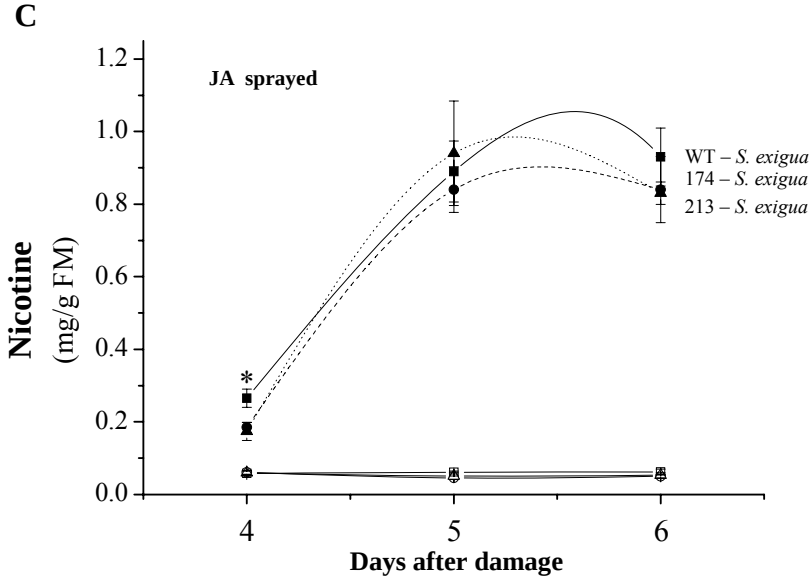

C
JA sprayed
Nicotine
(mg/g FM)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
4
5
6
Days after damage
*
WT – S. exigua
174 – S. exigua
213 – S. exigua

Figure 6. Silencing Na-*NPR1* influences nicotine accumulation in plants attacked by *S. exigua* larvae or sprayed with exogenous jasmonic acid. The values are the means (± SE) of total nicotine accumulated after different treatments. **A)** *S. exigua*-damaged leaves harvested 1, 2, and 3 days after the herbivore took its first bite, **B)** three-day-old *S. exigua*-damaged leaves sprayed with water and **C)** three-day-old *S. exigua*-damaged leaves sprayed with 1 mM JA. In treatments **B)** and **C)** leaves were harvested 4, 5, and 6 days after the herbivore took its first bite. Asterisks indicate that WT *S. exigua*-damaged plants differ significantly from both ir-*npr1* lines (174 and 213) (*, $P < 0.05$, **, $P < 0.01$, N=5).

These results demonstrate that the inability of ir-*npr1* plants to accumulate nicotine following *S. exigua* damage can be restored by JA treatment and we infer that JA limits nicotine accumulation in ir-*npr1* plants.

Silencing Na-*NPR1* reduces levels of OS-elicited JA and JA-Ile/Leu, but not ethylene bursts

Manduca sexta larvae attack and OS elicitation are known to elicit transient increases in JA and JA-Ile/Leu levels which are substantially larger than the increases that occur after identical amounts of wounding (Kang *et al*., 2006). These increases, referred to collectively as the oxylipin burst, are known to be elicited by the FACs in OS. Typically the burst attains maximum values within 45 min and subsequently declines rapidly (Halitschke and Baldwin, 2003; Roda *et al*., 2004). Feeding *S. exigua* larvae elicited significantly higher JA levels (> 4-fold) in WT plants compared to ir-*npr1* lines (174 and 213) (Fig. 7A; ANOVA, $F_{8, 18} = 287.4$, $P < 0.001$). Similar results were observed after treatment with *S. exigua* OS. W+OS-treated WT plants had higher JA levels (3-fold) than did plants of both ir-*npr1* lines (174 and 213) 45 minutes after OS elicitation (Fig. 7A; ANOVA, $F_{8, 18} = 6.9$, $P < 0.001$). The low JA burst in ir-*npr1* lines was not confined to the OS-elicited response: JA levels in the W+W treatment of WT plants were also significantly higher (1.4-fold) than those of ir-*npr1* lines (174 and 213) (Fig. 7; ANOVA, $F_{8, 18} = 3.21$, $P = 0.035$). Trends in the levels of JA-Ile/JA-Leu were similar to those in the levels of JA. In response to feeding *S. exigua*, WT plants accumulated more JA-Ile/JA-Leu (1.0-fold) than did either ir-*npr1* line (Fig. 7B; ANOVA, $F_{8, 18} = 8.63$, $P < 0.001$). Similar results were found in response to OS elicitation: WT plants accumulated more (1.0-fold) than did either ir-*npr1* line (Fig. 7B; ANOVA, $F_{8, 18} = 10.3$, $P < 0.0001$). The analysis of leaf samples from field-grown WT plants and ir-*npr1* line 213 also revealed that Na-*NPR1* silencing is correlated with reduced JA accumulation; ir-*npr1* line 213 accumulated less JA (85%) than did WT plants 45 minutes after OS elicitation (Fig. S7; ANOVA, $F_{17, 36} = 23.44$, $P < 0.001$). That JA and JA-Ile/Leu levels are severely

diminished in ir-*npr1* lines (174 and 213) suggests that Na-NPR1 exerts a major influence on the JA pathway and that JA is the limiting factor for the production of JA-Ile/Leu. In addition, we also measured ethylene 5 h after *S. exigua* attack and OS elicitation. In WT and ir-*npr1* plants, no significant differences were found between either *S. exigua*-damaged (Fig. S8; ANOVA, $F_{2, 6} = 0.211$, $P = 0.815$) or OS-elicited leaves (Fig. S8; ANOVA, $F_{2, 6} = 1.885$, $P = 0.231$). However, ethylene emission was significantly greater from *S. exigua*-attacked WT and ir-*npr1* plants than from undamaged WT and ir-*npr1* plants (Fig. S8; ANOVA, $F_{5, 12} = 8.68$, $P = 0.02$). Similarly, OS-elicited WT and ir-*npr1* leaves released significantly more ethylene compared to W+W-elicited WT and ir-*npr1* leaves (Fig. S8; ANOVA, $F_{5, 12} = 11.69$, $P < 0.001$). These results clearly demonstrate that Na-NPR1 does not influence the herbivore-induced ethylene burst, while it strongly influences the oxylipin burst.

Na-*NPR1* silencing increases SA levels in response to *S. exigua* attack and OS elicitation

NPR1 regulates SA levels in *Arabidopsis* (Shah, 2003) and our analysis revealed that the same holds for *N. attenuata*. In WT *N. attenuata* plants, free SA increased 45 minutes after *S. exigua* attack and OS elicitation, and levels remained elevated for 135 minutes. Interestingly, free SA levels were significantly higher (85%) 45 minutes after *S. exigua* damage in both ir-*npr1* lines (174 and 213) than in WT plants (Fig. 7C; ANOVA, $F_{8, 18} = 4.17$, $P = 0.005$). Similarly, 45 minutes after OS elicitation, plants from both ir-*npr1* lines (174 and 213) accumulated significantly more free SA (60%) than did WT (Fig. 7C; ANOVA, $F_{8, 18} = 3.582$, $P = 0.011$). Field-grown ir-*npr1* line 213 also had higher (1.0-fold) free SA levels compared to WT plants 45 minutes after OS elicitation (Fig. S7; ANOVA, $F_{17, 36} = 7.33$, $P < 0.0001$). Silencing Na-*NPR1* increases free SA, indicating that Na-NPR1 is likely a negative regulator of free SA production. These increases in free SA could result from increases in *de novo* synthesis and/or from increases in the release of free SA from SA conjugates. Because a majority of the SA in *Nicotiana* species occurs as sugar esters (Malamy *et al.,* 1992; Lee and Raskin, 1998), we also measured SA conjugates. Levels of conjugated SA also increased in OS-elicited WT compared to unelicited WT plants after 45 minutes, and levels in ir-*npr1* lines attacked by *S. exigua* were higher (60%) than in similarly damaged WT plants (Fig. S9; ANOVA, $F_{8, 18} = 3.30$, $P = 0.016$). OS-elicited ir-*npr1* lines also accumulated higher levels of conjugated SA (> 4-fold) than did similarly elicited WT plants (Fig. S9; ANOVA, $F_{8, 18} = 2.9$, $P = 0.025$).

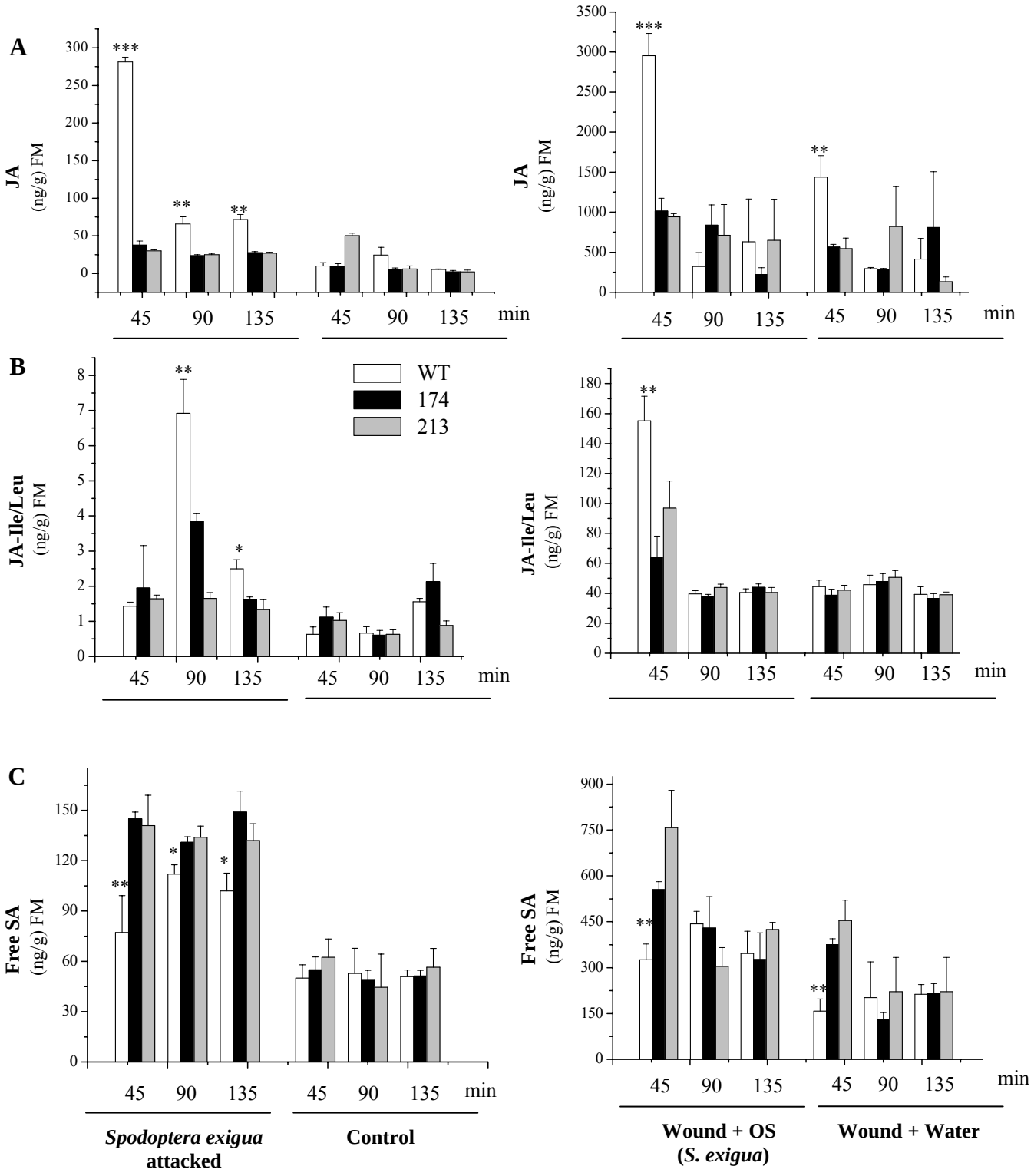


Figure 7. Silencing Na-*NPR1* reduces levels of JA and JA-Ile/Leu but increases free SA in plants attacked by *Spodoptera exigua* larvae (left) or elicited by W+OS or W+W (right) treatments in glasshouse-grown plants. **A**) Mean (± SE) JA (upper panel), **B**) JA-Ile/Leu (middle panel), and **C**) free SA (bottom panel) in leaves of 3 replicate ir-*npr1* (lines 174 and 213) and WT plants per genotype and treatment. Node +1 leaves were wounded with a fabric pattern wheel and the resulting puncture wounds immediately treated with 20 µl *S. exigua's* OS or water (W). For plants in the *S. exigua* treatments, 2 larvae were placed in a clip cage on each node +1 leaf.

Asterisks indicate significant differences between WT and both ir-*npr1* lines at the designated time (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, N=7).

Given that Na-NPR1 silencing increased both free and conjugated SA levels with similar kinetics, it is likely that *de novo* SA biosynthesis rather than its release from conjugated pools is responsible for the changes observed in SA levels. To better understand the role of SA biosynthesis, we analyzed transcripts of JA and SA biosynthetic genes.

Na-NPR1 modulates LOX 3 and ICS transcript accumulation

We conducted a qRT-PCR analysis of Na-*LOX3*, which codes for an enzyme catalyzing the oxygenation of linolenic acid at the 13-C position in the JA biosynthetic pathway, and of Na-*ICS*, an enzyme catalyzing the conversion of chorimate to isochorismate, which ultimately forms SA. Na-*LOX3* transcript levels in Na-*NPR1*-silenced lines (174 and 213) were significantly reduced (40% and 45%, respectively) compared to their levels in WT plants 1 h after *S. exigua* attack (Fig. 8A; ANOVA, $F_{11, 24} = 5.86$, $P = 0.0001$). In contrast, Na-*ICS* transcript levels in Na-*NPR1*-silenced lines (174 and 213) increased dramatically (nearly 2.5- and 3-fold, respectively; Fig. 8B; ANOVA, $F_{11, 24} = 8.16$, $P < 0.001$). These results demonstrate that Na-*NPR1* negatively regulates SA biosynthesis after herbivore attack. Interestingly, higher levels of Na-*ICS* transcripts were found in unattacked control WT plants (1.2-fold) than in WT plants that were attacked by *S. exigua*, which is consistent with a role for Na-NPR1 as a negative regulator of herbivore-induced SA biosynthesis.

Transcriptional responses of Na-*NPR1*-silenced plants to *S. exigua* attack

To understand how *S. exigua*-induced transcriptional responses are altered in *NPR1*-silenced plants (lines 174 and 213), we performed microarray analysis with a custom microarray enriched in *M. sexta*-induced *N. attenuata* genes (Halitschke and Baldwin, 2003; (Voelckel and Baldwin 2004). We hybridized arrays using RNA extracted from ir-*npr1* (lines 174 and 213) and WT plants that had been continuously attacked by *S. exigua* larvae for 24 h. For each of the two microarrays hybridized for each genotype, RNA was extracted from three biological replicate plants of WT plants and ir-*npr1* lines (174 and 213). Both *S. exigua*-damaged ir-*npr1* lines (174 and 213) were significantly altered in their expression of 47 and 41 genes, respectively.

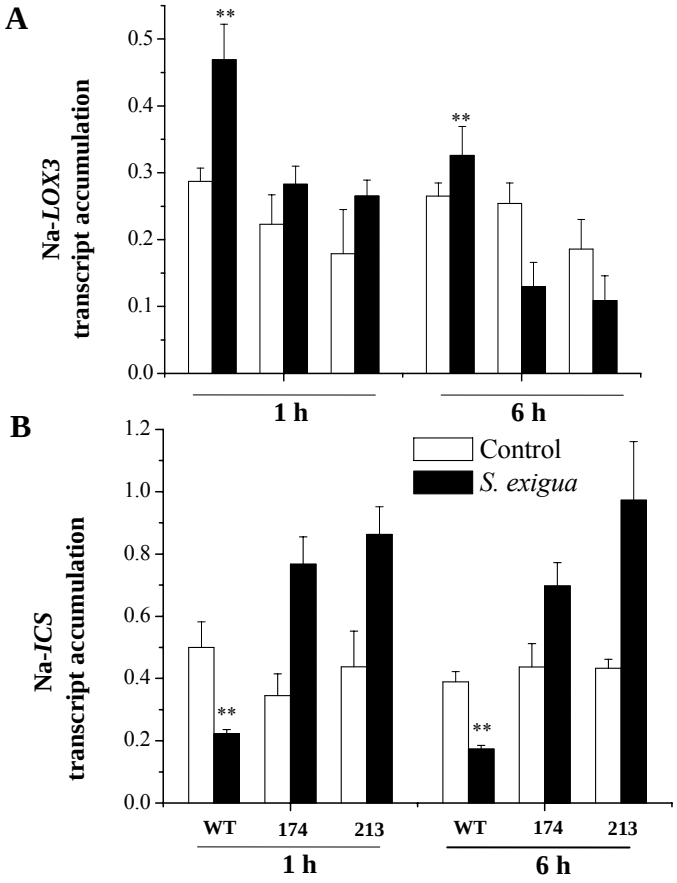


Figure 8. Na-*LOX3* and Na-*ICS* transcript accumulation in response to *S. exigua* damage in wild-type (WT) and ir-*npr1* lines (174 and 213). A first-instar larva previously reared on WT *N. attenuata* plants was enclosed in a well-aerated clip cage of diameter 5 cm. These clip cages were attached to the +1 nodal leaves of 6 replicate WT plants and ir-*npr1* lines (174 and 213). Leaves from 3 replicate plants of WT and ir-*npr1* lines (174 and 213) were harvested 1 and 6 h after the herbivore took its first bite. As a control, leaf tissues were harvested similarly from WT plants and ir-*npr1* lines (174 and 213) attached with the clip cage with no larvae inside. **A)** Na-*LOX3* (required for JA production) is up-regulated after herbivory in WT but not in ir-*npr1* lines. **B)** Na-*ICS* (required for SA production) is highly up-regulated in ir-*npr1* lines. The transcripts were analyzed by quantitative real-time PCR (qRT-PCR) and expressed as the mean (± SE) of 3 replicate leaves in arbitrary units from 100 ng cDNA prepared from RNA samples extracted 1 and 6 h after continuous feeding by *S. exigua* larvae. A constitutively unregulated sulfite reductase gene (*ECI*) was used for normalization. Asterisks represent significant differences between WT induced and ir-*npr1* induced lines (174 and 213), (*, $P < 0.05$; **, $P < 0.01$, N=3).

The summary of genes differentially regulated in both chips is presented in Fig. S10. *ICS* (*isochorismate synthase*) was up-regulated in both ir-*npr1* lines (174 and 213), a result consistent with the RT-PCR results, as were some genes involved in phenylalanine biosynthesis (DHQS), suggesting a regulatory role for Na-NPR1 with the phenylpropanoid pathway. JA-responsive genes such as *TPI* (*trypsin protease inhibitor*), *TPS* (*terpene synthase*, involved in sesquiterpene biosynthesis), and *ODC* (*ornithine decarboxylase*,

involved in nicotine production) were down-regulated in ir-*npr1* lines (174 and 213) but not in WT plants. These results are consistent with the observed changes in the levels of direct and indirect metabolites in ir-*npr1* plants and clearly demonstrate that Na-NPR1 negatively regulates JA-dependent defense responses. During herbivore damage, WT *N. attenuata* plants down-regulate photosynthetic genes such as *RUBISCO* and *PSII,* perhaps to allocate more resources to the production of defense compounds. Surprisingly, we found that *RUBISCO* and *PSII* were more strongly down-regulated in ir-*npr1* lines, suggesting that Na-NPR1-mediated responses might influence growth-related traits. A number of cytochrome P450 genes (CYP 71D2, CYP 81E8) known to be expressed during plant stress were down-regulated in ir-*npr1* lines. The transcriptional responses revealed by the microarray analysis suggest that Na-NPR1 may be involved in fine-tuning responses to herbivores by influencing genes that are SA- and JA-dependent as well as growth- and photosynthesis-related.

Discussion

In order to understand NPR1's influence on *N. attenuata's* direct and indirect defenses against herbivores, we compared the performance of herbivores feeding on WT and ir-*npr1* plants and correlated the results with the production of defense metabolites and with changes in the two main phytohormones (SA and JA) that mediate SAR and IR. We conducted this study in both *N. attenuata*'s natural habitat as well as under controlled glasshouse conditions. The results demonstrate that in addition to its well-established role in SAR, Na-*NPR1* influences the genes involved in IR. This was evident when Na-*NPR1*-silenced plants were transplanted into native habitats and found to be more susceptible than WT plants to naturally occurring herbivores as well as to *Pseudomonas* pathogens. The greater susceptibility to herbivores could be attributed to *N. attenuata's* impaired ability to elicit indirect (*cis*-α-bergamotene release) and direct (nicotine) defense responses. As a result, ir-*npr1* plants are less able to attract *G. pallens* predators and less able to increase nicotine levels. Both predator attraction and nicotine accumulation are known to be elicited by JA signaling. Exogenous JA application to nicotine-deficient ir-*npr1* plants restored normal nicotine levels. Field- and glasshouse-grown ir-*npr1* plants were found to accumulate high levels of SA and low levels of JA in response to herbivore attack and OS elicitation; these responses in phytohormone levels were mirrored by changes in the transcript levels of JA and SA biosynthesis genes. We propose that Na-NPR1 suppresses SA accumulation during

herbivore attack, which minimizes SA-JA antagonism and allows for the unhampered activation of JA-mediated defense responses.

The mechanisms of *N. attenuata's* IR have been well studied, and the importance of LOX3-mediated JA signaling in the production of several secondary metabolites involved in direct and indirect defenses are known (Halitschke and Baldwin, 2003; Steppuhn *et al.,* 2004; Zavala *et al.,* 2004). The increased susceptibility of ir-*npr1* plants to *S. exigua* is correlated with reduced levels of at least three secondary defense metabolites (nicotine, caffeoyl putrescine, and rutin). Although how caffeoyl putrescine and rutin function as defenses is unknown, the defensive function of nicotine has been established. Reduced nicotine levels are correlated with increases in *S. exigua's* performance on ir-*npr1* plants, and previous studies found *S. exigua* larvae to be the most significant lepidopteran herbivore on nicotine-silenced *N. attenuata* plants (ir-*pmt*) planted into a native population (Steppuhn *et al*., 2004). The release of JA-dependent *cis*-α-bergamotene, a VOC known to attract *G. pallens* predators to *M. sexta* eggs and early instar larvae (Kessler and Baldwin, 2001; Halitschke and Baldwin, 2003), is also impaired in ir-*npr1* plants, and as a result ir-*npr1* plants attract fewer predators after OS elicitation. Taken together, these results demonstrate that ir-*npr1* plants have a "defenseless" phenotype similar to that of JA-deficient plants (Kessler *et al*. 2004) probably due to increased SA production. Given that JA treatment can restore nicotine (a JA-dependent metabolite) production in *S. exigua* damaged ir-*npr1* plants, we propose that this "defenseless" phenotype results from SA-JA antagonism.

The effects of SA treatment on JA-mediated defenses are commonly interpreted as evidence of SA-JA antagonism. When SA, its methyl ester (MeSA), or SA mimics are applied to wounded or herbivore-attacked plants, the JA burst, JA-mediated gene expression, levels of JA-elicited defensive metabolites, as well as resistance to some herbivores are suppressed (Stout, Fidantsef et al. 1999; Thaler 1999). Comparable examples of such suppression can be found in the eicosanoids of animals, which are derivatives of C20:4 fatty acids. The eicosanoids share biosynthetic and structural similarities with the jasmonates, which are synthesized from 18:3 fatty acids. The cyclooxygenase enzymes of animals, like the LOXs of plants, are inhibited by salicylates, the best studied of which is acetylsalicylate (aspirin) (Vane, 1971). Suppressed JA signaling in Arabidopsis plants mutated in *mpk4* can be partially attributed to the plants' high SA levels (Wiermer *et al.,* 2005). In WT *Arabidopsis,* SA is thought to antagonize JA signaling during pathogen infection, which is corroborated by the diminishment of this antagonism in pathogen-elicited SA-deficient *NahG* plants that have high levels of *LOX2* transcripts and JA (Spoel *et al.,* 2003).

The SA-JA antagonism is not apparent in Arabidopsis *npr1* and *nim1* mutants in the Col-0 and Ws genetic backgrounds, respectively, but these plants do show evidence of altered JA-ethylene signaling when ISR is triggered (Pieterse *et al.,* 1998). ISR, which requires both JA and ethylene signaling, is SA-independent but NPR1-dependent. *Npr1-nim1* mutants tend to be more resistant to lepidopteran herbivores (*T. ni, S. littoralis, S. exigua*) perhaps due to their elevated levels of JA-inducible glucosinolates (Stotz *et al.,* 2002; Cipollini *et al.,* 2004; Mewis *et al.,* 2005), but whether the resistance results from altered SA-signaling or JA-ethylene signaling is not clear (von Poecke, 2007).

After herbivore attack or OS elicitation, ir-*npr1 N. attenuata* plants accumulate higher levels of SA and SA-biosynthetic transcripts but release ethylene in quantities similar to those in WT plants. Moreover, ir-*npr1* plants have diminished herbivory- and OS-elicited JA bursts, as well as low levels of JA biosynthetic transcripts and JA-mediated defenses (Figs. 7, 8, S6, S7, and S8). These results are consistent with the view that generalist herbivores, such as *S. littoralis*, may activate the SA pathway concomitantly with the JA pathway, perhaps to weaken JA-mediated resistance by amplifying the SA-JA antagonism (Stotz *et al.,* 2002; Cipollini *et al.,* 2004). In *Nicotiana sylvestris*, MeSA application reduces elicited nicotine accumulation (Baldwin *et al*., 1996; Baldwin *et al.,* 1997). In *Nicotiana tabacum,* TMV-inoculated plants (which are associated with local and systemic increase in endogenous SA) attenuated wound-induced JA and nicotine responses. Moreover, larvae consumed 1.7 to 2.7 times more leaf tissue from TMV-inoculated plants than from mock-inoculated plants (Preston *et al*., 1999).

Here we show that in *N. attenuata,* Na-*NPR1* silencing dramatically increases levels of free SA and reduces nicotine accumulation following herbivory. How herbivore attack elicits increases in SA levels remains an open question. Perhaps pathogenic factors in the larval OS activate an SA-dependent pathway, just as FACs activate JA signaling. Given that trade-offs between herbivore and pathogen resistance are likely to take place in plants (Bostock, 2005), and generalist herbivores are capable of activating SA-dependent responses, NPR1 may function as a regulatory protein capable of controlling SA production.

NPR1 occurs as a single copy gene in the *N. attenuata* genome and is clearly responsive to SA (Fig. 1). Among the different NPRs studied to date in *Arabidopsis*, Na-NPR1 is most similar (50%) to *Arabidopsis thaliana* NPR1 (At-NPR1) (Fig. S13). Since Na-*NPR1*-silenced plants were susceptible to both pathogens and herbivores (Fig. 3), the same Na-NPR1 is likely to function in SAR as well as to control SA production during IR. Apart from inhibiting the JA pathway, high SA levels are also associated with stunted growth

(Mauch, Mauch-Mani et al. 2001; Shah 2003). Although ir-*npr1* plants grew at normal rates in the glasshouse, microarray analysis revealed their *RUBISCO* and *PSII* transcripts were lower than those of OS-elicited WT plants. Since field grown ir-*npr1* plants tended to be slightly smaller than WT plants (although statistically not significant) (Fig. S.14), Na-NPR1 may influence growth, but additional experiments are needed to understand Na-NPR1's role in growth.

We conclude that the simultaneous activation of multiple signaling pathways involving SA and JA in plants can inhibit the activation of defense responses. To negate these effects, plants have evolved regulatory proteins such as NPR1 which help fine-tune defense responses by controlling SA production and thereby retain the function of the JA pathway.

Experimental procedures

Plant material, treatments and insect rearing

Wild-type (WT) *N. attenuata* plants selfed for 14 generations (seeds collected from a native population from the DI Ranch, Santa Clara, UT, USA) and ir-*npr1* lines 174 and 213, in which a 335 bp fragment of Na-*NPR1* is expressed in an inverted repeat orientation in the same WT genotype, were used in the experiment. Germination was carried out according to the procedures described by Krügel *et al.* (2002). For glasshouse studies, experiments were carried out on rosette-stage plants 13 days after they were transferred to 1 L pots.

For JA and SA treatments in the complementation studies, aqueous solutions 1 mM of JA (Sigma Chemical Co., St. Louis, MO, USA) and SA (Sigma Chemical Co., St. Louis, MO, USA) were used. JA was first dissolved in a small amount of ethanol and made up to a concentration of 1 mM with distilled water. SA and JA were sprayed on the entire plant, except for one leaf, till run-off occurred. Each leaf received approximately 400-500 µl of the solution. No phytotoxic effects were observed between unsprayed and sprayed leaf.

Neonate larvae of *Spodoptera exigua* (Lepidoptera: Noctuidae) hatched from eggs supplied by the Plant Protection Centre of Bayer AG (Monheim, Germany) were maintained in well-aerated plastic boxes (10 x10 cm) lined with filter paper to reduce humidity (photoperiod: 14–16 h photophase) at 22°C to 24°C . The larvae were fed an artificial diet consisting of 300 g /L agar, 400 g /L bean flour, 3 g sodium ascorbate, 3 g ethyl p-hydroxybenzoate, and 1 g formaldehyde. Small cubes of the diet were placed in rearing plastic boxes on pieces of aluminum foil. For the second experiment, larvae were maintained

similarly except that they were fed WT *N. attenuata* leaves. Eggs of *Manduca sexta L.* (Lepidoptera: Sphingidae), from North Carolina State University (Raleigh, NC, USA), were incubated at 26°C until they hatched, and caterpillars were fed WT *N. attenuata* plants and used for OS collection.

Isolating Na-*NPR1*

A 335 bp fragment of the genomic *NPR1* gene of *N. attenuata* was amplified using primers derived from conserved cDNA regions of *N. tabacum NPR1* (Liu *et al.,* 2002) using a forward primer *NPR1* 5 (3'-CCTGATAAACATGTTAAGAGG-5') and a reverse primer *NPR1* 6 (3'- GCCTAGTGAGCCTCTTGGC -5') (Fig. S1). Using *N. tabacum* as the reference sequence (Liu *et al.,* 2002), a full-length ORF was isolated. Two sets of primers were used: 1) forward primer FL3P (3'-ATGGATAATAGTAGGACTGCG -5') and reverse primer RL5P (3'-TCTTCTTTCTGCTTGCTC-5'); and 2) forward primer OLF1 (3'-CAGTAAGTCTCCAGAGGAAGGA-5') and reverse primer OLR1 (3' CTATTTCCTAAAAGGGAGCTT-5') (Fig. S1). Using these two sets of primers, a 1767 bp full-length ORF was PCR-amplified from cDNA prepared from RNA extracted from W+OS (*M. sexta*)-elicited *N. attenuata* leaves. The fragments were excised from the gel, purified using an Amersham gel purification kit (Buckinghamshire, UK), and cloned in a pGEM-T Easy vector (Promega, Madison, WI, USA) following the manufacturer's instructions. Positive colonies were picked by blue-white screening and grown overnight, and the plasmid was isolated with a Macheray and Nagel kit (Duren, Germany). The fragments were sequenced and compared with the known sequences from the NCBI database which confirmed the clone to be Na-*NPR1*.

Nucleotide sequences of the ORF of Na-*NPR1* were aligned with the Megalign (DNASTAR, Madison, WI, USA) program. To determine the phylogenetic relationships among the different *NPR1* sequences from the NCBI database, we used the neighbor-joining method with bootstrap analysis (1000 replicates) (Wu *et al.,* 2006).

Generation and characterization of Na-*NPR1*-silenced plants (ir-*npr1*)

A 335 bp fragment of the cDNA sequence of Na-*NPR1* was inserted into the pRESC5 transformation vector as an inverted-repeat construct (Fig. S3). This vector was transformed into *N. attenuata* WT plants using an *Agrobacterium*-mediated transformation procedure previously described in Krügel *et al.* (2002). The gene for hygromycin resistance (*hptII*) allowed transformed plants to be identified by selecting hygromycin-resistant

individuals (Krügel *et al.,* 2002). Southern hybridization of genomic DNA from independently transformed T2 generation plants and from WT lines was carried out using a PCR fragment of the *hptII* gene as a probe. Lines harboring a single copy of the transgene resulting from independent transformation events were identified and further screened for homozygosity after they were germinated on GB-5 germination plates supplemented with hygromycin. Two independently transformed single-insert homozygous lines with 100% germination on hygromycin-containing GB5 Petri plates, strongly suppressed in their Na-*NPR1* transcript accumulation (see Fig. S4, A and B) and with WT growth morphologies, were used for all further experiments (lines 174 and 213).

Nucleic acid analysis

DNA was extracted from the leaf tissue of fully developed plants using the cetyl-trimethylammonium bromide (CTAB) method originally developed by Rogers and Bendich (1985) and modified by Paschold *et al.* (2007): for the Southern blot hybridizations, 15 µg of the DNA samples was digested with different restriction enzymes at 37°C overnight, separated on a 0.8% (w/v) agarose gel, and Southern-blotted onto a nylon membrane (GeneScreen Plus, Perkin Elmer, Rodgau-Jügesheim, Germany). The 335 bp fragment of Na-*NPR1* was isolated (see above), and an *hptII* probe (forward primer: 5'-CGTCTGTCGAGAAGTTTCTG-3' and reverse primer: 3'- CCGGATCGGACGATTGCG-5') generated by PCR amplification were used as probes for Southern hybridization to confirm the *NPR1* copy number and single insertion transgenic lines, respectively. Both probes were labeled with α-^{32}P (Rediprime™ II DNA labeling system, Amersham Biosciences, Freiburg, Germany).

To analyze Na-*NPR1* transcripts, we extracted total RNA with TRI reagent following the TIGR protocol (http://www.tigr.org/tdb/potato/images/SGED_SOP_3.1.1.pdf). cDNA was synthesized from 1 µg RNA using SuperScript™ II RT (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR (ABI PRISM™7000, Applied Biosystems) was conducted using the qPCR™ core reagent kit (Eurogentec, Seraing, Belgium), a Na-*NPR1*-specific TaqMan primer pair (forward primer: 5'-GTGTCCCTTTTAACCAAAGGA-3', the reverse primer: 5'- GCAGATTTTCCTTCCTCT -3'), and a double fluorescent dye-labeled probe (5'-CATCCGATGGCAGAAAAGCACTTCAAA-3'). The relative gene expression was calculated using a 10-fold dilution series of cDNAs containing Na-*NPR1* transcripts of *sulfite reductase* (*ECI*), which are not regulated under our experimental conditions (B. Bubner and I.

T. Baldwin, unpublished data); these served as endogenous control genes (Bubner and Baldwin 2004).

To analyze Na-*NPR1* transcript accumulation in transgenic plants, 1 mM SA was sprayed on leaves until run-off. Tissues were harvested 1 h after elicitation (N=3). A similar procedure was employed to analyze the kinetic of Na-*NPR1* regulation in response to attack from *S. exigua* larvae*,* except that the leaf samples were from *S. exigua*-attacked plants which had not been treated with SA. To examine Na-*LOX3* transcript accumulation in *S. exigua*-damaged tissues of WT plants and ir-*npr1* lines, we used a Na-*LOX3*-specific TaqMan primer pair (forward primer: 5'-GGCAGTGAAATTCAAAGTAAGAGC-3', reverse primer: 5'-CCCAAAATTTGAATCCACAACA-3'), and a double fluorescent dye-labeled probe (5'-CAGTGAGGAACAAGAACAAGGAAGATCTGAAG-3'). For isochorismate synthase (Na-*ICS*) we used a SYBR-green based RT-PCR approach, with Na-*ICS1*-specific primers (forward primer: 5'-TTTGCAACCTCCCCAGTC-3', reverse primer: 5'-ACCCCTAGCCCGTGTTC- 3').

Western blot analysis

To isolate polyclonal antibodies against Na-NPR1, a 15-amino-acid peptide was synthesized using the cDNA sequence of Na-*NPR1* (N'-**CKG/ARP/SDL/TSD/GRK**--C'). This peptide was used to immunize a rabbit and antiserum against the synthesized peptide and obtained after 10 weeks (Genemed Synthesis, San Francisco, CA, USA). Protein samples were separated on an 8.0% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h at room temperature in TTBS buffer (12.5 mM Tris–HCl, 137 mM NaCl, 0.1% Tween 20, pH 7.5) containing 5% non-fat dried milk (TTBS), washed three times in TTBS, and incubated for 1 h at room temperature in 1:5000–1:10 000 dilutions of Na-NPR1 anti-serum in TTBS. Blots were washed in TBS-T buffer three times and incubated for 1 h at room temperature in a 1:10000 dilution of goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (GeneScreen Plus, Perkin Elmer, Rodgau-Jügesheim, Germany) in TBS-TMIL buffer. Antibody-bound proteins were visualized after the blots were incubated with alkaline phosphatase buffer containing 17 µl NBT and 17 µl BCIP for 30 min.

Direct defense traits

M. sexta and *S. exigua* oral secretions (OS) were collected from 3rd and 4th instar larvae. OS were diluted 1:2 (v:v) with deionized water before being added to puncture wounds. Leaf tissue (100-150 mg) for the analysis of nicotine, rutin, and caffeoyl putrescine was sampled 24, 48, and 72 h after elicitation by treating puncture wounds with water or the OS of *S. exigua.* On separate plants, leaf tissues (100-150 mg) were sampled 24, 48, and 72 h after *S. exigua* began feeding. Secondary metabolites that are strongly correlated with resistance to *S. exigua* in *N. attenuata* were analyzed by HPLC as described in Steppuhn *et al.* (2004). Leaf samples (~100 mg) from *S. exigua*- or W+OS-elicited (+1 nodal leaves) glasshouse-grown plants, 5 replicates for each genotype were extracted with 2:3 methanol: 0.5% acetic acid (v/v) and analyzed by HPLC-DAAD. A standard curve was made using a dilution series of nicotine and rutin, which was used to calculate the amounts of nicotine and rutin. In the case of caffeoyl putrescine, for which synthetic standards are not commercially available, the amounts were expressed as relative peak areas. For all metabolites, quantities were normalized to the exact amount of tissue used for the extraction.

Analysis of JA, JA-amino acid conjugates, SA, and ethylene

About 200 mg of frozen tissue samples was finely ground and transferred to FastPrep tubes containing 0.9 g of FastPrep matrix. One ml of 90% MeOH spiked with 200 ng ^{13}C-JA and pCA was added to each sample before samples were homogenized on a FastPrep homogenizer (Thermo Electron) and centrifuged at 6000g for 15 min at 4°C. The supernatants were transferred to fresh 2.0 ml Eppendorf tubes and 1 ml of 100% MeOH was added for re-extraction. The combined supernatants were dried in a vacuum at 45°C after drying, 1 ml of hot water (80°C) was added. The samples were divided into 2 500µl aliquots to measure free and conjugated SA, JA, and JA-Ile/Leu; 500 µl of 0.2 M acetate buffer was added to the samples of free SA, JA, and JA-Ile/Leu, and 0.2 M acetate buffer containing 0.1 mg/ml β-glucosidase was added to the sample for conjugated SA analysis. Both samples were incubated at 37°C for 14 h, after which the samples' pH was adjusted to 1-1.5. 700 µl of cyclopentane/ethyl acetate/isopropanol (50:50:1) was gently added and the organic extract was separated and dried under nitrogen. Finally, the dried samples were suspended in 70% MeOH and pipetted to new glass vials before being analyzed by a 1200L LC/MS-MS-MS system (Varian, Palo Alto, CA, USA). The instrument was set with a flow rate of 0.1 ml/min, and 15 µL of each sample was injected onto a Pursuit C8 column (3 µm, 150 × 2 mm) (Varian). A mobile phase composed of solvent A (0.05% formic acid) and solvent B (0.05% formic acid in methanol) was used in a gradient mode for separation. Phytohormones were

detected in negative ESI mode as described in Wang *et al.* (2007). JA and its conjugates were estimated based on the peak area of the internal standard; SA was estimated based on a standard curve from the serial dilution of SA. For ethylene, the exact protocol as described in von Dahl *et al*. (2007) was used. In brief, +1 leaves of three pairs of WT and ir-*npr1 lines* (174 and 213) plants were treated with OS (of *S. exigua*) on the mechanically wounded leaves and immediately sealed in a 250 ml 3-necked flask. To analyze ethylene from the *S. exigua*-damaged leaves, three pairs of WT and ir-*npr1* plants (lines 174 and 213) were fed on by 2 *S. exigua* larvae; after they took their first few bites, leaves were sealed in a 250 ml 3-necked flask. Ethylene was allowed to accumulate in the flask for 300 min. The headspace was flushed into a photoacoustic laser spectrometer (Invivo GmbH, Saint Augustin, Germany) with hydrocarbon-free air. Ethylene concentrations were measured by comparing ethylene peak areas with the peak generated by a standard ethylene gas.

Analysis of herbivory

Plants were grown in the glasshouse (16/8 hr photoperiod at 200-300 μmol m^{-2} s^{-1}, 25/21°C, and 45-55% relative humidity) in 1 L pots with soil. To analyze *S. exigua's* growth performance on glasshouse-grown plants, we placed 3-day-old larvae that had been reared on WT *N. attenuata* leaves on the fully developed leaves of rosette-stage WT and ir-*npr1* plants (N=15). Each larva was enclosed in a well-aerated clip cage of diameter 5 cm. The larvae were weighed 3, 6, and 9 days after feeding. We then repeated the assay with larvae that had been reared on artificial diet (see above).

Performance under field conditions

WT and transgenic ir-*npr1* lines were planted into the natural habitat of *N. attenuata* in the southwestern United States. Seeds of WT and ir-*npr1* plants were germinated on agar plates. The plates were incubated at 25°C/16 h (200 μm/s/m^2 light) and 20°C/8 h dark. After 10 days, seedlings were transferred to Jiffy 703 pots (1 ¾ inch x 1 ¾ inch, AlwaysGrows, Newark, OH, USA) which had been soaked in borax solution (0.4 mg/ 45 ml water). The seedlings were fertilized with iron solution (stock solution: 2.78 g $FeSO_4.7H_2O$ and 3.93 g Titriplex in 1 L H_2O, diluted 100-fold for fertilization) after 7 days. Seedlings were allowed to gradually adapt to the environmental conditions of the Great Basin Desert (high sun exposure and low relative humidity) over 2 weeks in a mesh tent before being transplanted into the field sites. Plants were transplanted in size-matched pairs to an irrigated field plantation at the Lytle Ranch Preserve (Santa Clara, UT) and into native *N. attenuata*

populations in a blackbrush and pinyon-juniper forest that had burned in 2005. Seventeen ir-*npr1*-WT pairs of size-matched acclimated seedlings were planted in transects at the burn site. Seedlings were watered every other day for 2 weeks until roots were established in the native soil. The release of transgenic plants was carried out under APHIS notification (06-003-08n). To comply with the 7CFR 340.4, the legal statute which governs the release of transgenic organisms, plants were either harvested and destroyed before the start of flowering (burn site) or flowers were removed before seeds matured (plantation site).

We analyzed the total herbivore damage 21 days after transplanting into the field sites. We estimated the percentage of leaf area removed (in the case of grasshoppers), or the percentage of characteristic damage caused by specific herbivores/pathogens relative to the total leaf area as described in Paschold *et al.* (2007). Damage was expressed as the percentage of canopy damage/plant after dividing the total percentage of damage by the total number of leaves.

Pathogen identification

We collected leaves infected by naturally occurring pathogens from the field and cultured them on LB media. Single colonies distinct from other colonies in color and morphology were picked and re-plated. Five isolates were purified from the infected leaves. Plates containing single colonies were sent to AMODIA (Braunschweig, Germany), for identification. A single colony from five different plates was sequenced for the 16S ribosomal RNA to identify the pathogen. Three isolates had 16S sequences with more than 98% similarity to those of *Pseudomonas spps* and other two isolates matched the isolate of *Pantoea sps* (Table S1).

Indirect defenses

We used a *M. sexta* egg predation assay to measure how well *N. attenuata*'s herbivore-induced VOCs attract the dominant predator of *N. attenuata's* herbivores, *Geocoris pallens* (Kessler and Baldwin 2002). Using a natural cellulose glue which is known to have no effect on the predation rate or the plants' VOC emissions, we glued five *M. sexta* eggs to the abaxial side of the second stem leaf of seven matched pairs of ir-*npr1* and WT plants, 30 days after the plants had been planted into the field. We counted the number of predated *M. sexta* eggs (which are transparent and papery) 12, 20, 38, and 42 h after they were glued to the leaf. We elicited the first stem leaves with W+OS 42 h after the eggs were glued and measured egg predation again 17, 20, and 25 h after elicitation.

Analysis of volatile organic compounds (VOCs)

To trap VOCs released by OS elicitation, we used mechanically punctured leaves of field-grown plants and immediately treated the puncture wounds with *M. sexta* OS. Since the OS of both *M. sexta* and *S. exigua* contain common FACs (Voelckel and Baldwin 2004), it's possible to mimic each herbivore's response. We enclosed the OS-elicited leaves individually in open-ended polystyrene chambers and trapped VOCs at approximately 350 ml/min for 8 h with charcoal traps (Orbo M32, Supelco, Munich, Germany) as described in Kessler and Baldwin (2001). After the experiment the charcoal traps were stored at -20°C until VOC elution and analysis. Before eluting the VOCs for GC-MS analysis, the traps were spiked with 80 ng tetraline as an internal standard and eluted with 500 µL dichlormethane as described by Paschold *et al.* (2007).

Oligonucleotide-microarray analysis

A customized microarray containing 50-mer oligonucleotides from 1,404 herbivore-regulated genes was designed by spotting each oligo four times onto epoxy-coated glass slides (Quantifoil Microtools, Jena, Germany) (Voelckel and Baldwin, 2004a, 2004b). First-instar *S. exigua* larvae were placed on the second fully expanded (+1) leaves of five replicate glasshouse-grown WT and ir-*npr1* plants (lines 174 and 213). Leaf tissue was harvested from the damaged leaves and from separate control plants at the same positions. cDNA from damaged leaves of one genotype was Cy3-labeled and cDNA from the corresponding control leaves from the same genotype was Cy5-labeled. A competitive hybridization was performed on the gene-spotted epoxy slides. Each hybridization was replicated twice. An Affymetrix 428TM array scanner (Affymetrix, Santa Clara, CA, USA) was used to scan the microarrays sequentially for Cy3- and Cy5-labeled cDNA at a maximum resolution of 10 µm/pixels with a 16-bit depth. The final data were statistically analyzed using a lowess-normalization procedure with the MIDAS package (TIGR microarray data analysis system, Institute for Genome Research, Washington, DC, USA). For a gene to qualify as up- or down-regulated, a minimum of a 1.5-fold change in expression ratio was required as well as a *t-test* at confidence level (α) 0.05 for the quadruplicate spots of each gene. A gene was regarded as differentially regulated if it met both criteria in both microarrays from each line. In some cases, a gene was also defined to be significantly regulated when the signal of the gene was present in only one channel and the density was more than 2.5-fold the signal-to-noise ratio.

Statistical analysis

Data were analyzed with StatView (Abacus Concepts, Inc., Berkeley, CA, USA). One-way ANOVAs with Bonferroni-corrected post-hoc tests were used to analyze the data.

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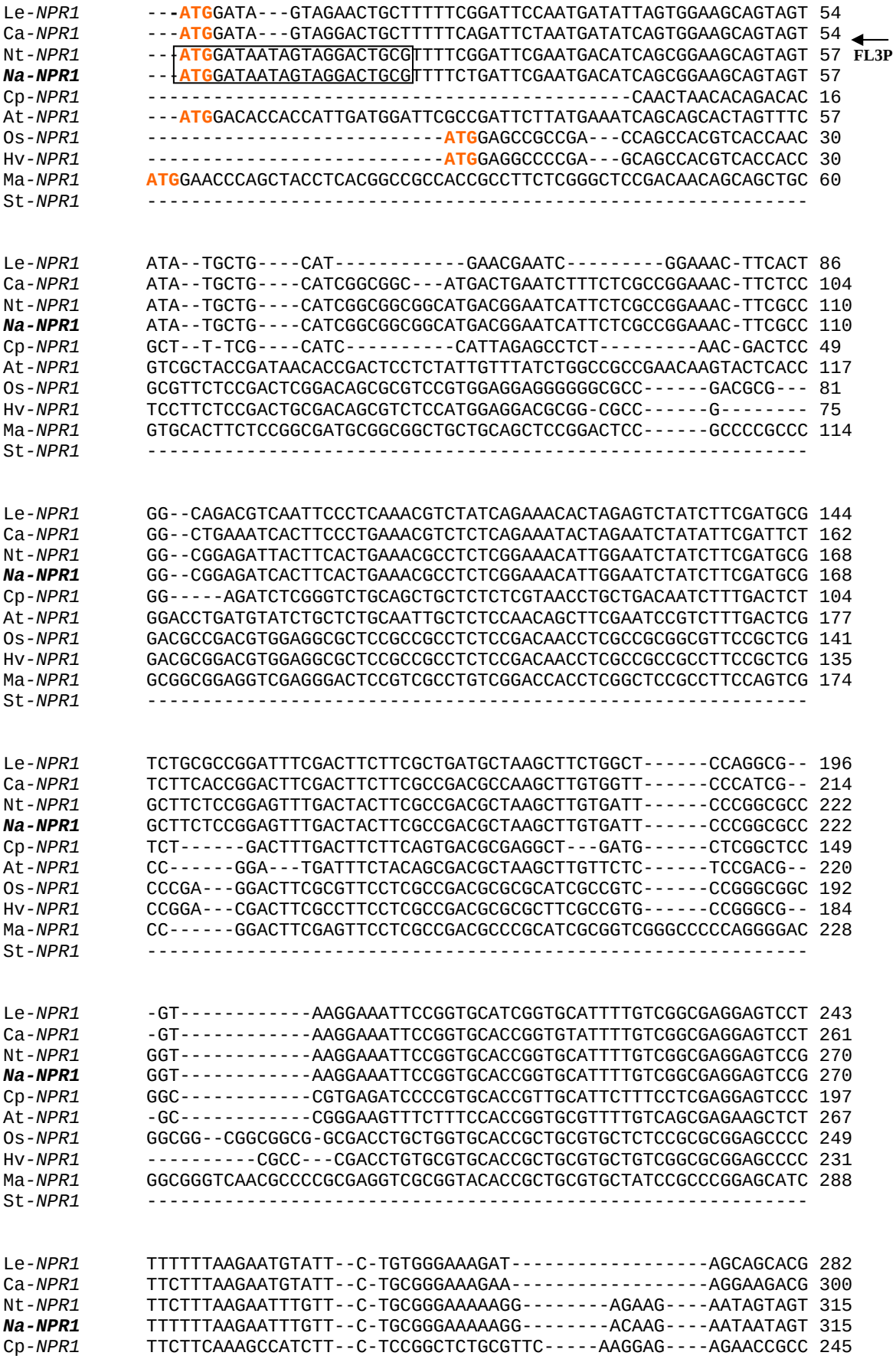
Supplemental Figures



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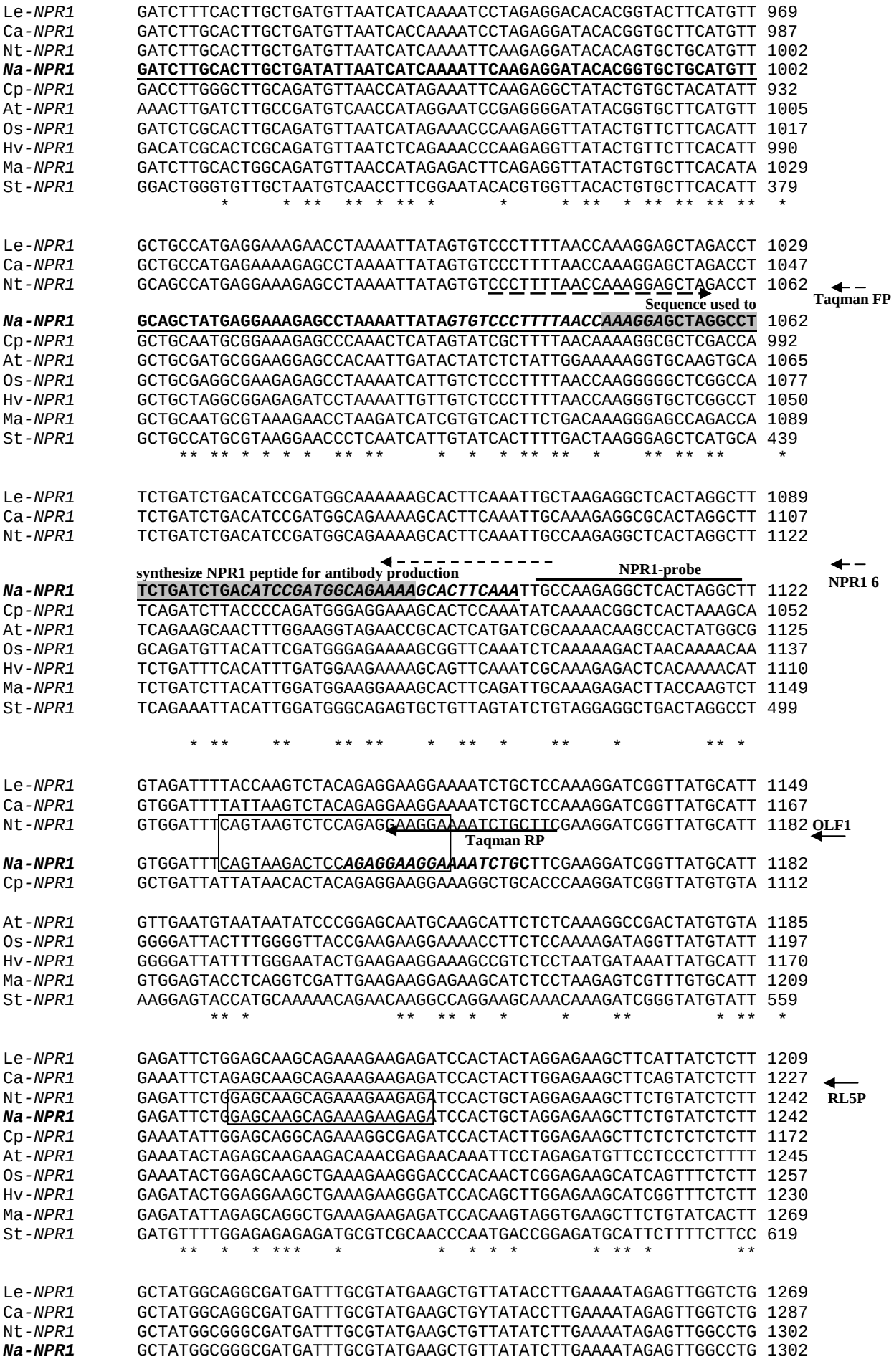
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GCAGCCATGAGGAAAGAGCCTAAAATTATAGTGTCCCTTTTAACCAAAGGAGCTAGACCT 1062
Sequence used to
Taqman FP
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Os-NPR1 GCTGCGAGGCGAAGAGAGCCTAAAATCATTGTCTCCCTTTTAACCAAGGGGGCTCGGCCA 1077
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Ma-NPR1 GCTGCAATGCGTAAAGAACCTAAGATCATCGTGTCACTTCTGACAAAGGGAGCCAGACCA 1089
St-NPR1 GCTGCCATGCGTAAGGAACCCTCAATCATTGTATCACTTTTGACTAAGGGAGCTCATGCA 439
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TCTGATCTGACATCCGATGGCAGAAAAGCACTTCAAATTGCAAAGAGGCGCACTAGGCTT 1107
TCTGATCTGACATCCGATGGCAGAAAAGCACTTCAAATTGCCAAGAGGCTCACTAGGCTT 1122
synthesize NPR1 peptide for antibody production
NPR1-probe
NPR1 6
Na-NPR1 TCTGATCTGACATCCGATGGCAGAAAAGCACTTCAAATTGCCAAGAGGCTCACTAGGCTT 1122
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Ma-NPR1 TCTGATCTTACATTGGATGGAAGGAAAGCACTTCAGATTGCAAAGAGACTTACCAAGTCT 1149
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Taqman RP
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Os-NPR1      GCAATGGCAGGTGAGAGTCTACGAGGAAGGTTGCTGTATCTTGAAAACCGAGTTGCTTTG 1317
Hv-NPR1      GCATTGGCTGGTGACTGCCTTCGGGGGAAGTTATTGTACCTTGAAAACCGAGTGGCTTTG 1290
Ma-NPR1      GCAATGGCTGGTGATGACTTGCGGGGAAGATTGTTGTATCTTGAGAATCGAGTTGCTCTG 1329
St-NPR1      CCCATGTTGGCCGATGATCTGCCCATGAAACTGCTCTACCTGGAAAATAGAGTGGCATTT 679
                  *       *  **     *       *   *  *  * ** ** **  **** *   *

Le-NPR1      GCTAAACTCCTTTTTCCCATGGAAGCAAAAGTTGCAATGGACATTGCACAAGTTGATGGC 1329
Ca-NPR1      GCTAAACTCCTTTTCCCYATGGAAGCAAAAGTTGCAATGGACATTGCTCAAGTTGATGGC 1347
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Na-NPR1      GCTAAACTCCTTTTTCCAATGGAAGCAAAAGTTGCAATGGACATTGCTCAAGTTGATGGC 1362
Cp-NPR1      GCAAAACTTCTTTTCCCCATGGAAGCAAAAGTTGCAATGGATATTGCCCAAGTGAATGGA 1292
At-NPR1      GCTCAACGTCTTTTTCCAACGGAAGCACAAGCTGCAATGGAGATCGCCGAAATGAAGGGA 1365
Os-NPR1      GCGAGGATTATGTTTCCGATGGAGGCAAGAGTAGCAATGGATATTGCTCAAGTGGATGGA 1377
Hv-NPR1      GCGAGGATAATGTTTCCAATTGAGGCAAGAGTAGCAATGGACATTGCTCAGGTGGATGGT 1350
Ma-NPR1      GCAAGACTATTGTTCCCCATGGAGGCAAGAGTTGCTATGGACATTGCACAAGTTGATGGC 1389
St-NPR1      GCACGATTATTGGTCCCT---------------------------------------- 697
                 **        *  * **

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Le-NPR1      ACAGTGGATTTGAACGAGGCTCCTTTCAAGATGAAAGAGGAGCACTTGAATCGGCTTAGG 1446
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Na-NPR1      ACGGTAGATTTGAACGAGGTTCCTTTCAGGATAAAAGAGGAGCACTTGAATCGGCTTAGA 1479
Cp-NPR1      ACTATGGATTTGAATGAGGCGCCTTTCAGAATCCAAGAGGAGCACCTGAATAGACTCAGA 1400
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Hv-NPR1      ACCGTTGATCTAAATGATACTCCTTTCAAAATGAAGGATGAACACTTGGCTCGGATGAGA 1461
Ma-NPR1      GCGATGGATCTAAACGAAGCACCATTCAAGATCAAGGAAGAGCATCTGGCACGAATGAGA 1506
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Cp-NPR1      GCACTCTCTAGAACTGTGGAACTAGGGAAACGGTTTTTCCCTCGTTGTTCTGAAGTACTG 1460
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Cp-NPR1      AACAAAATCATGGATGCTGATGATTTGTCATTGCTTGCACGTCTGGAACATGATACCCCA 1520
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Ma-NPR1      AACAAGATCATGGA---CGACGATCTCACAGAAATCACTGGCCTCGGACACCACACTTCG 1623
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Ma-NPR1      GAGGAG---------AAGAGGAGGAGATTTCAGGAGTTGCAGGAAGTCCTGTCAAAAGCA 1674
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St-NPR1      ------------------------------------------------------------

Le-NPR1      -
Ca-NPR1      -
Nt-NPR1      -
Na-NPR1      -
Cp-NPR1      -
At-NPR1      -
Os-NPR1      -
Hv-NPR1      -
Ma-NPR1      A 1779
St-NPR1      -
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OLR1

Figure S1. Alignment of *N. attenuata NPR1* ORF (EF 441289-submitted) with *NPR1* ORF sequences from different plant species using the Clustal W method (http://www.ebi.ac.uk/clustalw). The regions for invert-repeat constructions and hybridization probes are underlined and set in bold face. Forward and reverse primers for the real-time PCR are denoted by arrows, and real-time PCR probe is denoted by a blunt arrow located between the primer sequences. Start- and stop-codons are set in bold face and orange font. Sequences that are framed in boxes represent primers (described in Experimental procedures) used to amplify NPR1 sequences in *N. attenuata* using the *N. tabacum* NPR1 sequence. Asterisks indicate nucleotide identity, and dashes, missing nucleotides (Le- *Lycopersicon esculentum* – AY 640378, Ca- *Capsicum annum* – DQ 648785, Nt- *Nicotiana tabacum* – AF 480488, Na- *Nicotiana attenuata* – submitted (EF 441289), Cp- *Carica papaya* – AY 548108, At- *Arabidopsis thaliana* – NM 105102, Os- *Oryza sativa* – DQ 450947, Hv- *Hordeum vulgare*- AM 050559, Ma- *Musa acuminata* - EF 137717, and St- *Solanum tuberosum* – AY 615281).

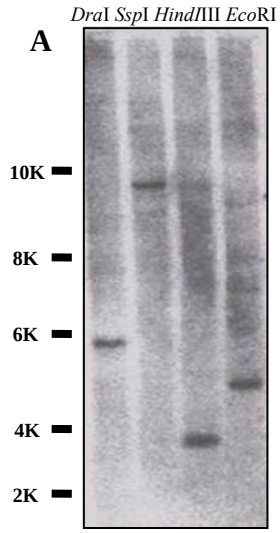

Figure S2.

Figure S2. Southern blot of Na-*NPR1* in WT plants. Ten µg of genomic DNA was digested with *Dra*I, *Ssp*I, *Hind*III and *Eco*RI. Digested DNA was blotted onto nylon membranes. Blots were hybridized with Na-*NPR1*-specific probe (see **Fig. S1**).

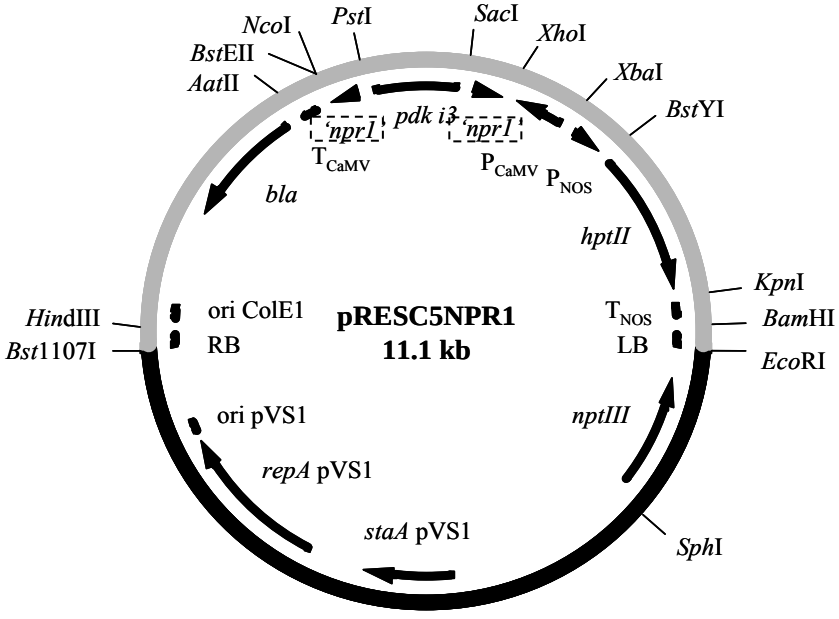

Figure S3. Transformation vector used to generate transgenic plants silenced in Na-*NPR1*. A 335 bp fragment of Na-*NPR1* was inserted twice in opposite directions, resulting in an inverted-repeat construct in the pRESC5 backbone vector.

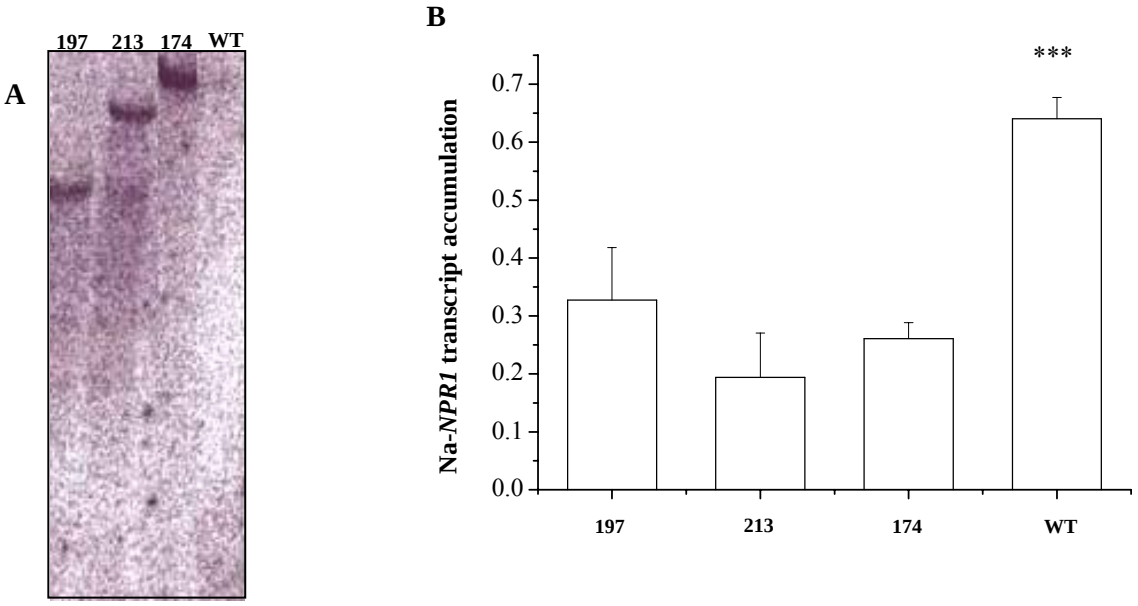

Figure S4. A) Southern blot of *NPR1*-silenced plants. Ten µg genomic DNA was digested with *Eco*RI and blotted onto nylon membranes and the blots were hybridized with a hygromycin (*hptII*) probe. The results confirm those of the segregation analysis: all transformed lines harbored a single copy of the transgene. **B)** Na-*NPR1* transcript accumulation 1h after SA treatment. Three replicate wild-type plants (WT) and three ir-*npr1* lines (197, 213, and 174) were sprayed with 1 mM SA. The transcript accumulation was quantified by real-time PCR (qRT-PCR) and expressed as the mean (± SE) of 3 replicate leaves in arbitrary units from 100 ng cDNA prepared from RNA extracted from three replicate plants per genotype and treatment. Asterisks represent significant differences between ir-*npr1* lines and WT plants (***, $P < 0.001$, N=3).

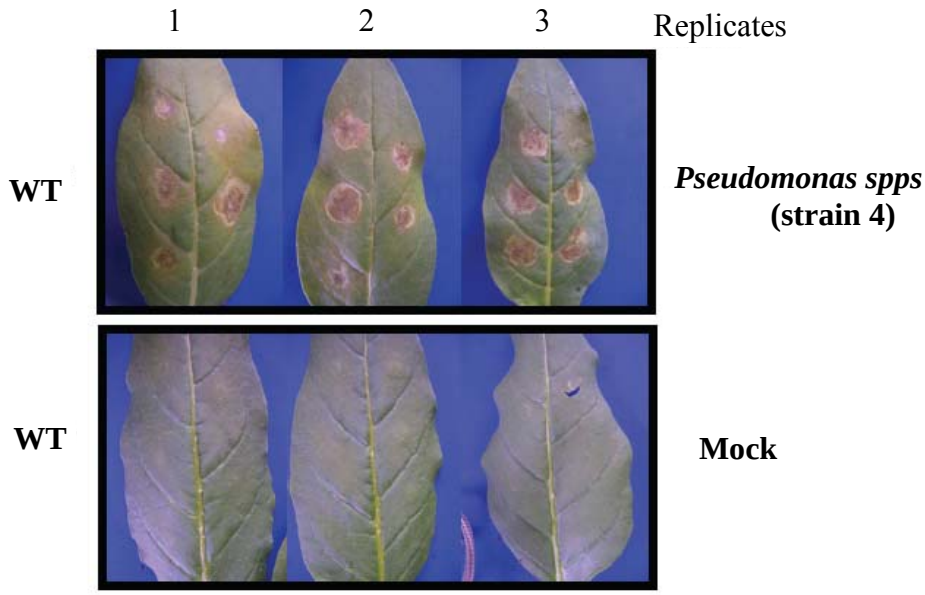

Figure S5. Disease symptoms (8 days post inoculation) caused by field-isolated *Pseudomonas sps* (strain 4) on WT *N. attenuata* under glasshouse conditions. A strain of *Pseudomonas spps* (strain 4) bacterial culture grown overnight was diluted to $1*10^5$ cells/ml of sterile water. Each leaf was injected at five spots (100 µl of bacterial culture/ spot) **(top panel)**. As a control 100 µl of sterile water/spot was injected **(bottom panel)**. In both treatments 3 replicate WT plants were used.

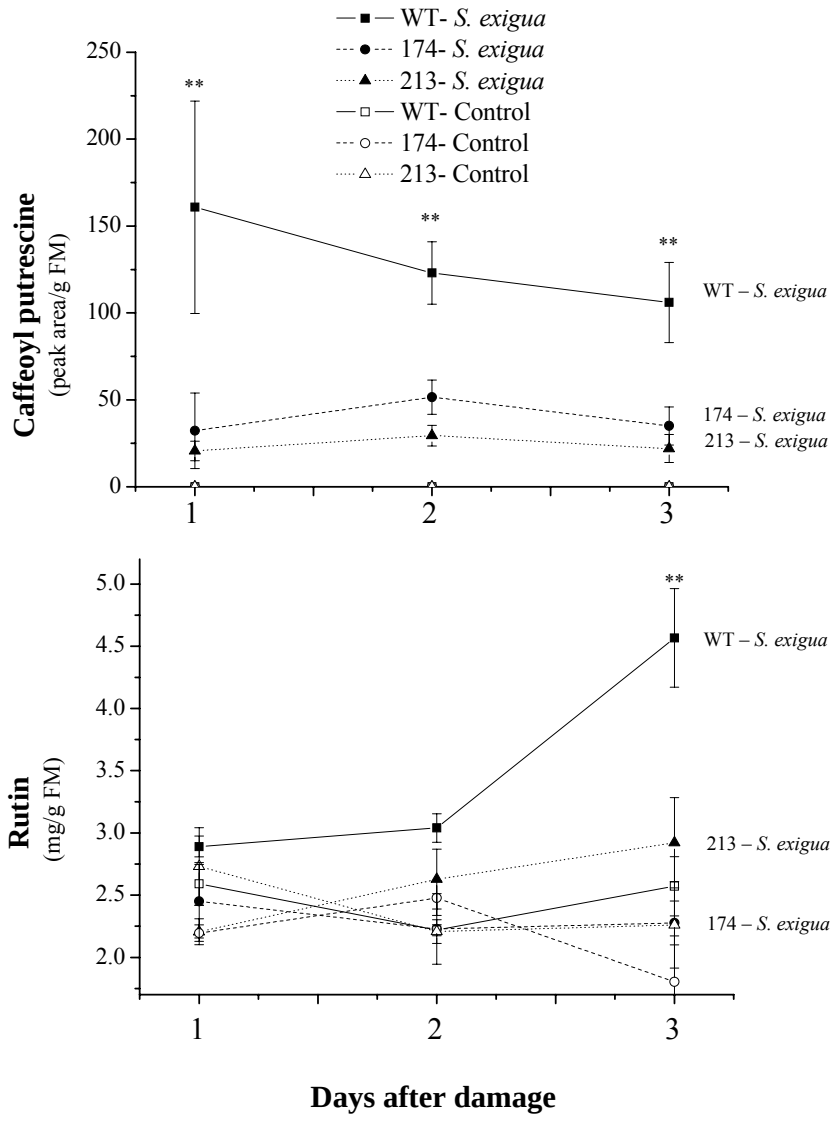

Figure S6. Influence of silencing Na-*NPR1* on rutin and caffeoyl putrescine accumulation in response to *S. exigua* damage. The values are the means (± SE) of 5 replicate plants per genotype and treatment. Asterisks indicate that WT *S. exigua*-damaged plants differ significantly from both ir-*npr1* lines (213 and 174) (*, $P < 0.05$; **, $P < 0.01$, N=5).

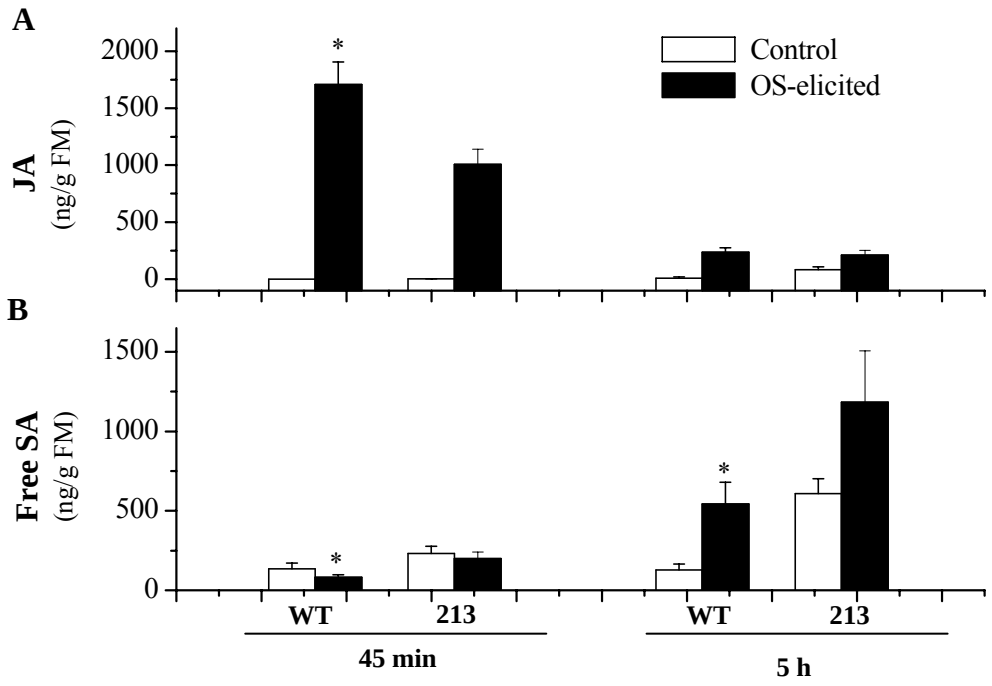

Figure S7. Silencing Na-*NPR1* reduces JA levels but increases levels of free SA in field-grown OS-elicited plants. Mean (± SE) JA (**A**) and free SA (**B**) in leaves of 5 replicate ir-*npr1* (213) and WT plants per genotype and treatment. Node +1 leaves were wounded with a fabric pattern wheel and the resulting puncture wounds immediately treated with 20 µl *M. sexta*'s OS or with water (controls). Asterisk indicates significant differences between induced WT and ir-*npr1* line (213) at the respective harvest times, $P < 0.05$ (N=5).

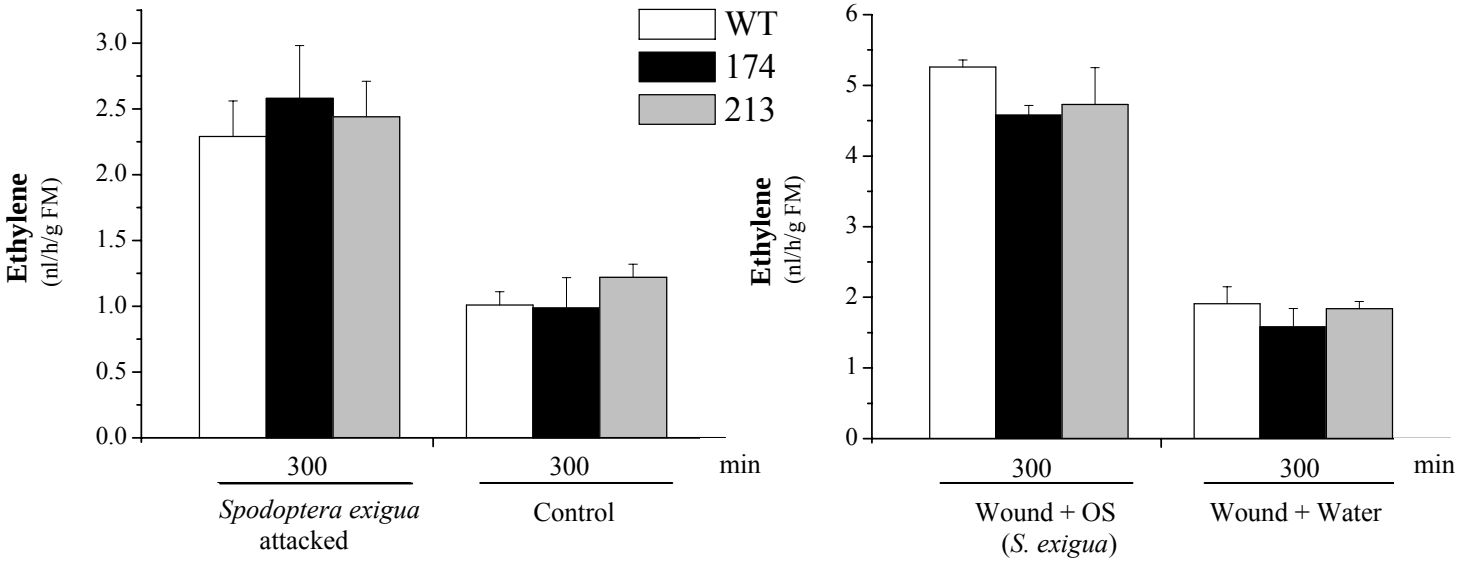

Figure S8. Silencing Na-*NPR1* did not affect the amount of ethylene released after herbivore damage (left) or after W+OS (right) elicitation in the leaves of ir-*npr1* (213 and 174) and WT glasshouse-grown plants. Node +1 leaves were wounded with a fabric pattern wheel and the resulting puncture wounds immediately treated with 20 µl *S. exigua* OS on 3 replicate plants per genotype and treatment. Ethylene from the headspace of the elicited leaves was collected for 5 h. For *S. exigua* insect elicitation, a single larva was placed on 3 replicate plants per genotype and treatment in a clip cage with a node +1 leaf inserted in it. Ethylene from the headspace of an *S. exigua*-damaged leaves was collected for 5 h after the herbivore took its first bite. The ethylene was analyzed using a photo acoustic spectroscopy (PAS) with a laser source tuned so as to excite ethylene molecules.

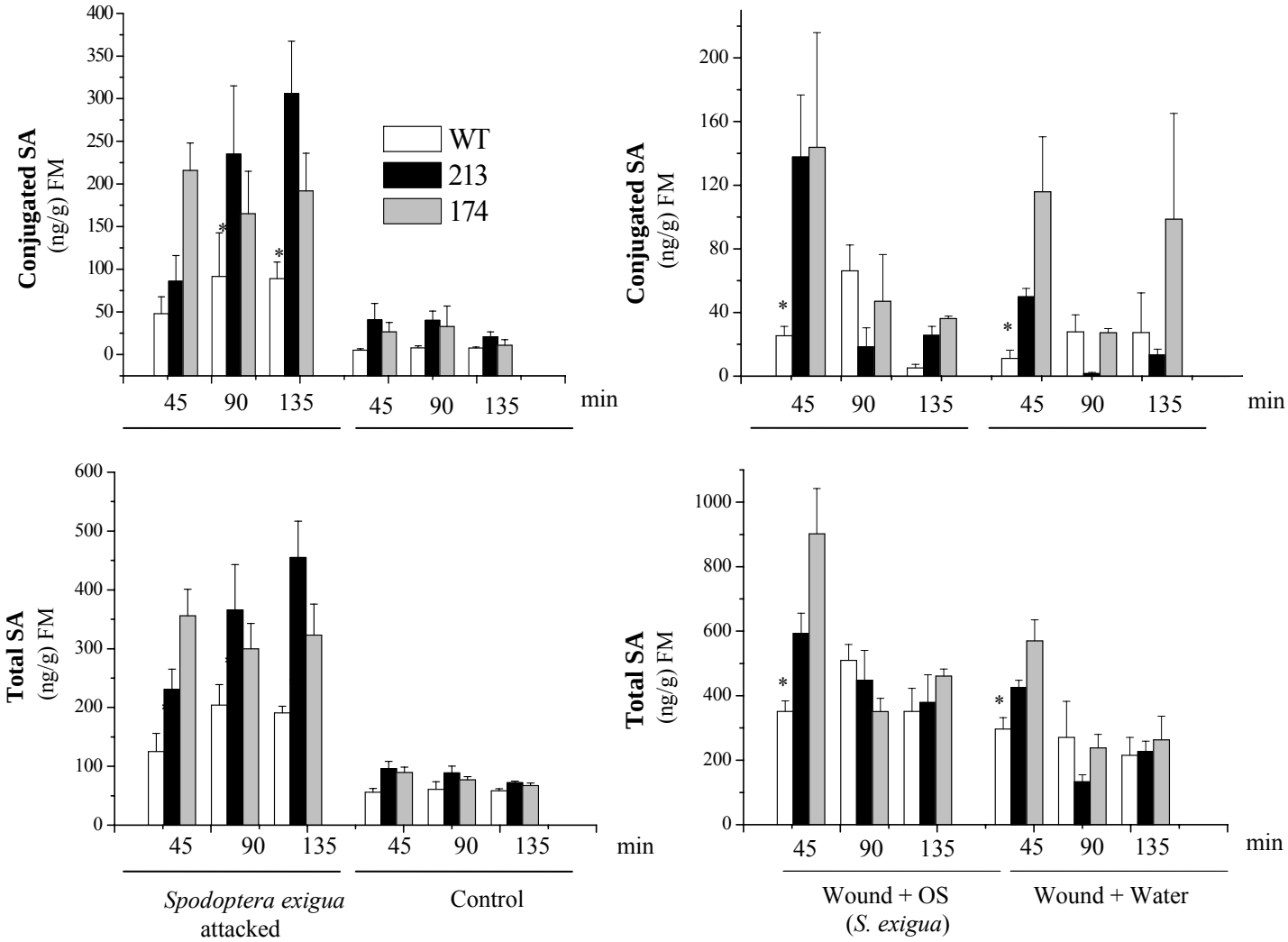

Figure S9. Silencing Na-*NPR1* increases levels of conjugated SA and total SA during continuous herbivory (left) as well as after W+OS (right) elicitation in the glasshouse. Mean (± SE) conjugated SA **(top panel)** and total SA **(bottom panel)** in leaves of ir-*npr1* (174 and 213) and WT plants. Node +1 leaves were wounded with a fabric pattern wheel and the resulting puncture wounds immediately treated with 20 µl *S. exigua* OS, 5 replicate plants per genotype and treatment. For *S. exigua* insect elicitation, 2 larvae were placed on a node +1 leaf on 5 replicate plants (per genotype and treatment) in a clip cage. Asterisk represents significant differences between WT and ir-*npr1* lines at $P < 0.05$, N=5.

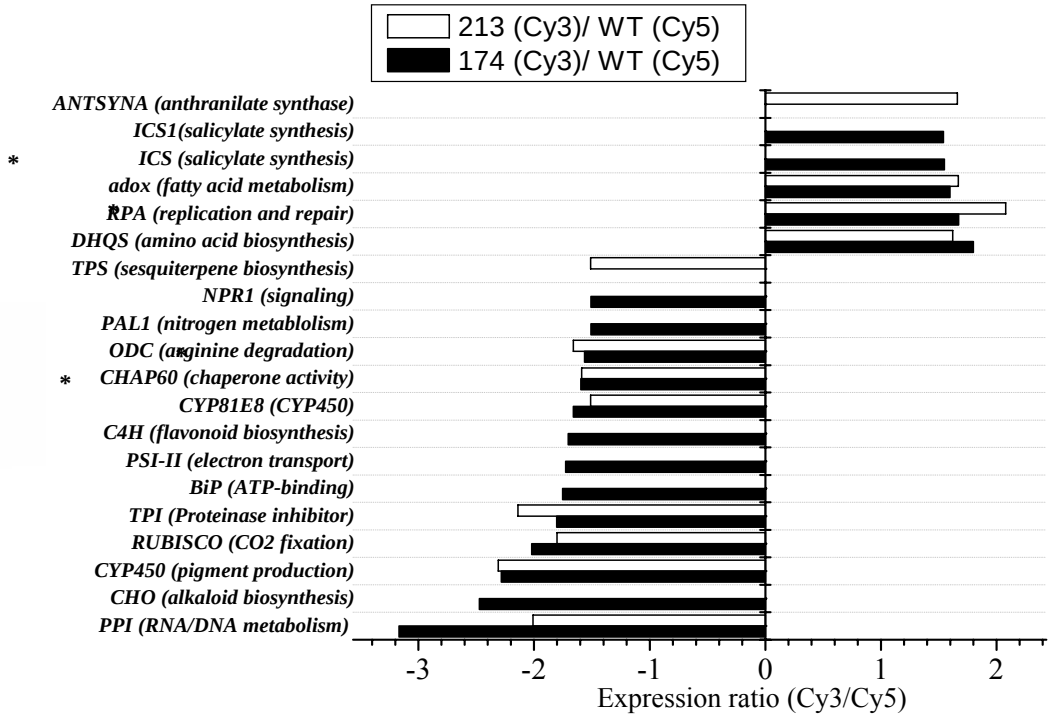

Figure S10. Silencing *NPR1* changes herbivory-elicited transcriptional responses. A microarray experiment was designed to analyze changes in the gene expression in *NPR1*-silenced plants when plants were damaged by *S. exigua* for 24 h. cDNA derived from *S. exigua*-damaged ir-*npr1* lines (174 and 213) labeled with Cy3 were hybridized against cDNA derived from similarly attacked WT *N. attenuata* plants labeled with Cy5. Two microarrays were hybridized for each line. The data were lowess-normalized with the MIDAS package (TIGR microarray data analysis system, Institute for Genome Research, Washington, DC, USA). A threshold of a 1.5-fold change in expression ratio and a *t-test* at confidence level (α) 0.05 were used to analyze the significance of the quadruplicate spots of each gene. A gene was regarded as differentially regulated if it met both criteria in both microarrays from each line. In some cases, a gene was also defined to be significantly regulated when the signal of the gene was present in only one channel and the density was more than 2.5-fold the signal-to-noise ratio (***** represents mean expression ratios that did not fulfill all the requirements).

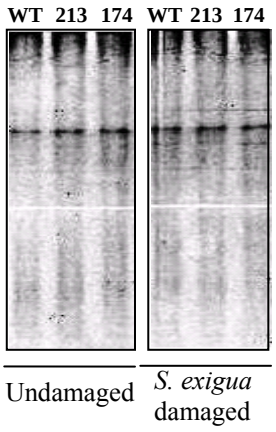

Figure S11. Loading controls for western blot analysis shown in Fig. 2B. Amounts of total proteins (20 µg) from WT and two ir-*npr1* lines (213 and 174) loaded and separated on an 8% SDS-PAGE gel used in the analysis depicted in Fig. 2B. Later the gel was stained with commassie blue.

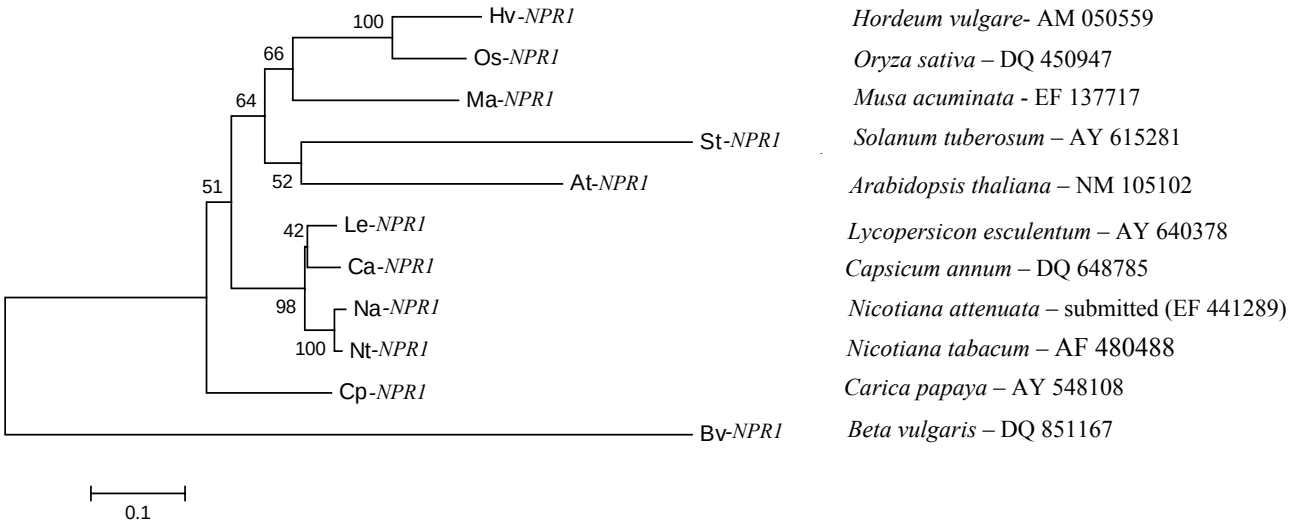

Figure S12. Neighbor-joining tree of *NPR1* ORF sequences. Nucleotide Kimura 2-parameters model and 1,000 bootstrap replicates were employed for tree construction. *NPR1* ORF sequences from different plant species were retrieved from the NCBI GeneBank. The ORF of Na-*NPR1* is submitted to NCBI gene bank under the accession number, EF441289**.**

A)


Na-NPR1 ----MDNSRTAFSDSNDISGSSSICCIGGGMTESFS---PETSPAEITSLKRLSETLESI 53
At-NPR1 ----MDTTIDGFADSYEISSTSFVATDNTDSSIVYLAAEQVLTGPDVSALQLLSNSFESV 56
At-NPR2 MATTTTTTTARFSDSYEFSNTSGNSFFAAESSLDYP--TEFLTPPEVSALKLLSNCLESV 58
At-NPR3 -----MATLTEPSSSLSFTSSHFSYGSIGSNHFSSS----SASNPEVVSLTKLSSNLEQL 51
At-NPR4 ----MAATAIEPSSSISFTSSHLSNPSPVVTTYHS------AAN-----LEELSSNLEQL 45
At-NPR5 ----------MSN-LEESLRSLSLDFLNL 18
At-NPR6 ----------MSNTFEESLKSMSLDYLNL 19
Na-NPR1 FDAASPEFDYFADAKLVIPGAGKEIPVHRCILSARSPFFKNLFCGKK--------DKNNS 105
At-NPR1 FDSPD---DFYSDAKLVLS-DGREVSFHRCVLSARSSFFKSALAAAK--------KEKDS 104
At-NPR2 FDSPE---TFYSDAKLVLA-GGREVSFHRCILSARIPVFKSALATVK--------EQKSS 106
At-NPR3 LSNSD---CDYSDAEIIVDG--VPVGVHRCILAARSKFFQDLFKKEK--------KISKT 98
At-NPR4 LTNPD---CDYTDAEIIIEEEANPVSVHRCVLAARSKFFLDLFKKDK--------DSSEK 94
At-NPR5 LINGQ------AFSDVTFSVEGRLVHAHRCILAARSLFFRKFFCGTDSPQPVTGIDPTQH 72
At-NPR6 LINGQ------AFSDVTFSVEGRLVHAHRCILAARSLFFRKFFCESDPSQP--GAEPANQ 71
Na-NPR1 ----------KVELKEVMKEYEVSYDAVVSVLAYLYSGKIRPSPKDVC---VCVDNDCSH 152
At-NPR1 NN----TAAVKLELKEIAKDYEVGFDSVVTVLAYVYSSRVRPPPKGVS---ECADENCCH 157
At-NPR2 -------TTVKLQLKEIARDYEVGFDSVVAVLAYVYSGRVRSPPKGAS---ACVDDDCCH 156
At-NPR3 E-------KPKYQLREMLPYGAVAHEAFLYFLSYIYTGRLKPFPLEVS---TCVDPVCSH 148
At-NPR4 --------KPKYQMKDLLPYGNVGREAFLHFLSYIYTGRLKPFPIEVS---TCVDSVCAH 143
At-NPR5 GSVPASPTRGSTAPAGIIPVNSVGYEVFLLLLQFLYSGQVSIVPQKHEPRPNCGERGCWH 132
At-NPR6 ---TGSGAR-AAAVGGVIPVNSVGYEVFLLLLQFLYSGQVSIVPHKHEPRSNCGDRGCWH 127
Na-NPR1 PAVAFLVEILYTSFTFQISELVDKFQRHLLDILGKTAADDVMVVLSVANICGKACE 212
At-NPR1 PAVDFMLEVLYLAFIFKIPELITLYQRHLLDVVDKVVIEDTLVILKLANICGKACM 217
At-NPR2 SKVDFMVEVLYLSFVFQIQELVTLYERQFLEIVDKVVVEDILVIFKLDTLCGTTYK 216
At-NPR3 PAIDFVVQLMYASSVLQVPELVSSFQRRLCNFVEKTLVENVLPILMVAFNC--KLT 206
At-NPR4 PAIDFAVELMYASFVFQIPDLVSSFQRKLRNYVEKSLVENVLPILLVAFHC--DLT 201
At-NPR5 AAVDLALDTLAASRYFGVEQLALLTQKQLASMVEKASIEDVMKVLIASRKQ--DMH 190
At-NPR6 AAVDLSLDILAAARYFGVEQLALLTQKHLTSMVEKASIEDVMKVLIASRKQ--DMH 185
Na-NPR1 RLLSSCIEIIVKSNVDIITLDKALPHDIVKQITNSRAELGLQ-----------GPESNG 260
At-NPR1 KLLDRCKEIIVKSNVDMVSLEKSLPEELVKEIIDRRKELGLE-----------VPK--- 262
At-NPR2 KLLDRCIEIIVKSDIELVSLEKSLPQHIFKQIIDIREALCLE-----------PPK--- 261
At-NPR3 QLLDQCIERVARSDLYRFCIEKEVPPEVAEKIKQLRLISPQDE---------ETSPKISE 257
At-NPR4 QLLDQCIERVARSDLDRFCIEKELPLEVLEKIKQLRVKSVN------------IPEVED 248
At-NPR5 QLWTTCSHLVAKSGLPPEILAKHLPIDVVTKIEELRLKSSIARRSLMPHNHHHDLSVAQD 250
At-NPR6 QLWTTCSYLIAKSGLPQEILAKHLPIELVAKIEELRLKSSMPLRSLMPH--HHDLTSTLD 243
Na-NPR1 FPDKHVKRIHRALDSDDVELLQMLLREGHTTLDDAFALHYAVAYCDAKTTAELLDLALAD 320
At-NPR1 -VKKHVSNVHKALDSDDIELVKLLLKEDHTNLDDACALHFAVAYCNVKTATDLLKLDLAD 321
At-NPR2 -LERHVKNIYKALDSDDVELVKMLLLEGHTNLDEAYALHFAIAHCAVKTAYDLLELELAD 320
At-NPR3 KLLERIGKILKALDSDDVELVKLLLTESDITLDQANGLHYSVVYSDPKVVAEILALDMGD 317
At-NPR4 KSIERTGKVLKALDSDDVELVKLLLTESDITLDQANGLHYAVAYSDPKVVTQVLDLDMAD 308
At-NPR5 LEDQKIRRMRRALDSSDVELVKLMVMGEGLNLDESLALHYAVESCSREVVKALLELGAAD 310
At-NPR6 LEDQKIRRMRRALDSSDVELVKLMVMGEGLNLDESLALIYAVENCSREVVKALLELGAAD 303
Na-NPR1 INHQN-SRGYTVLHVAAMRKEPKIIVSLLTKGARPSDLTSDGRKALQIAKRLTRLVDFSK 379
At-NPR1 VNHRN-PRGYTVLHVAAMRKEPQLILSLLEKGASASEATLEGRTALMIAKQATMAVECNN 380
At-NPR2 VNLRN-PRGYTVLHVAAMRKEPKLIISLLMKGANILDTTLDGRTALVIVKRLTKADDYKT 379
At-NPR3 VNYRN-SRGYTVLHFAAMRREPSIIISLIDKGANASEFTSDGRSAVNILRRLTNPKDYHT 376
At-NPR4 VNFRN-SRGYTVLHIAAMRREPTIIIPLIQKGANASDFTFDGRSAVNICRRLTRPKDYHT 367
At-NPR5 VNYPAGPAGKTPLHIAAEMVSPDMVAVLLDHHADPNVRTVGGITPLDILRTLTS------ 364
At-NPR6 VNYPAGPTGKTALHIAAEMVSPDMVAVLLDHHADPNVQTVDGITPLDILRTLTS------ 357

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Na-NPR1  TPEEGKSASKDRLCIEILEQAERRDPL-LGEASVSLAMAGDDLRMKLLYLENRVGLAKLL 438
At-NPR1  IPEQCKHSLKGRLCVEILEQEDKREQI-PRDVPPSFAVAADELKMTLLDLENRVALAQRL 439
At-NPR2  STEDGTPSLKGGLCIEVLEHEQKLEYLSPIEASLSLPVTPEELRMRLLYYENRVALARLL 439
At-NPR3  KTAKGRESSKARLCIDILEREIRKNPMVL-DTPMCSISMPEDLQMRLLYLEKRVGLAQLF 435
At-NPR4  KTSR-KEPSKYRLCIDILEREIRRNPLVSGDTPTCSHSMPEDLQMRLLYLEKRVGLAQLF 426
At-NPR5  ----------------------DFLFKGAVPGLTHIEPNKLRLCLELVQS-------- 392
At-NPR6  ----------------------DFLFKGAIPGLTHIEPNKLRLCLELVQS-------- 385
                               : :     .       :.*:: *   :.

Na-NPR1  FPMEAKVAMDIAQVDGTSEFP-LASIS-KKMVNAQRTTVDLNEVPFRIKEEHLNRLRALS 496
At-NPR1  FPTEAQAAMEIAEMKGTCEFI-VTSLEPDRLTGTKRTSPGVKIAPFRILEEHQSRLKALS 498
At-NPR2  FPVETETVQGIAKLEETCEFT-ASSLEPDHHIGEKRTSLDLNMAPFQIHEKHLSRLRALC 498
At-NPR3  FPTEAKVAMDIGNVEGTSEFTGLS--PPSSGLTGNLSQVDLNETPHMQTQRLLTRMVALM 493
At-NPR4  FPAEANVAMDVANVEGTSECTGLLTPPPSNDTTENLGKVDLNETPYVQTKRMLTRMKALM 486
At-NPR5  ------AAMVISREEGNNSNN------------QNNDNNTGIYPHMNEEHNSGSSGGSN 433
At-NPR6  ------AALVISREEGNNNS---------------NDNNTMIYPRMKDEHTSGSS---- 419
               ..  :.. . . .                    .    *    :.

Na-NPR1  RTVELGKRFFPRCSEVLNKIMD---ADDLSEIAYMGNDTAEERQLXKQRYMXLSEILTKA 553
At-NPR1  KTVELGKRFFPRCSAVLDQIMN---CEDLTQLACGEDDTAEKRLQKKQRYMEIQETLKKA 555
At-NPR2  KTVELGKRYFKRCS--LDHFMD---TEDLNHLASVEEDTPEKRLQKKQRYMELQETLMKT 553
At-NPR3  KTVETGRRFFPYGSEVLDKYMAEYIDDDILDDFHFEKGSTHERRLKRMRYRELKDDVQKA 553
At-NPR4  KTVETGRRYFPSCYEVLDKYMDQYMDEEIPDMSYPEKGTVKERRQKRMRYNELKNDVKKA 546
At-NPR5  NNLDSRLVYLNLGAGTGQMGPG----RDQGDDHNSQREGMSRHHHHHQDPSTMYHHHHQH 489
At-NPR6  --LDSRLVYLNLGATN---------RDIGDDNSNQREGMNLHHHHH-DPSTMYHHHHHH 466
           ::    ::                :  .          :   :     : .   :

Na-NPR1  FPEDKE-EFDKTN--NISSSCSSTSKGVDKPNKLPFRK---------------- 588
At-NPR1  FSEDNL-ELGNSSLTDSTSSTSKSTGGKRSNRKLSHRRR--------------- 593
At-NPR2  FSEDKE-ECGKS-------STPKPTSAVRSNRKLSHRRLKVDKRDFLKRPYGNGD 600
At-NPR3  YSKDKESKIARSC---LSASSSPSSSSIRDDLHNTT------------------ 586
At-NPR4  YSKDK---VARSC---LSSSS--PASSLREALENPT------------------ 574
At-NPR5  HF---------------------------------------------------- 491
At-NPR6  F----------------------------------------------------- 467
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B)

	***N. attenuata* NPR1**
***A. thaliana* NPR1**	50.1%
***A. thaliana* NPR2**	47.7%
***A. thaliana* NPR3**	39.7%
***A. thaliana* NPR4**	39.2%
***A. thaliana* NPR5**	20.2%
***A. thaliana* NPR6**	20.4%

Figure S13. A) Alignment of *N. attenuata* NPR1 (deduced amino acid) (EF 441289-submitted) with different NPRs of *Arabidopsis thaliana* using the Clustal W method (http://www.ebi.ac.uk/clustalw). Asterisks indicate amino acid identity; dashes, missing amino acids; and dots, amino acid variations. Na-NPR1 (submitted) (EF 441289), At-NPR1- NM 105102, At-NPR2- At 4g26120, At-NPR3- At5g45110, At-NPR4- At4g19660, At-NPR5- At2g41370, At-NPR6- At3g57130. B) Percent amino acid sequence identity of Na-NPR1 with all known At-NPR1s.

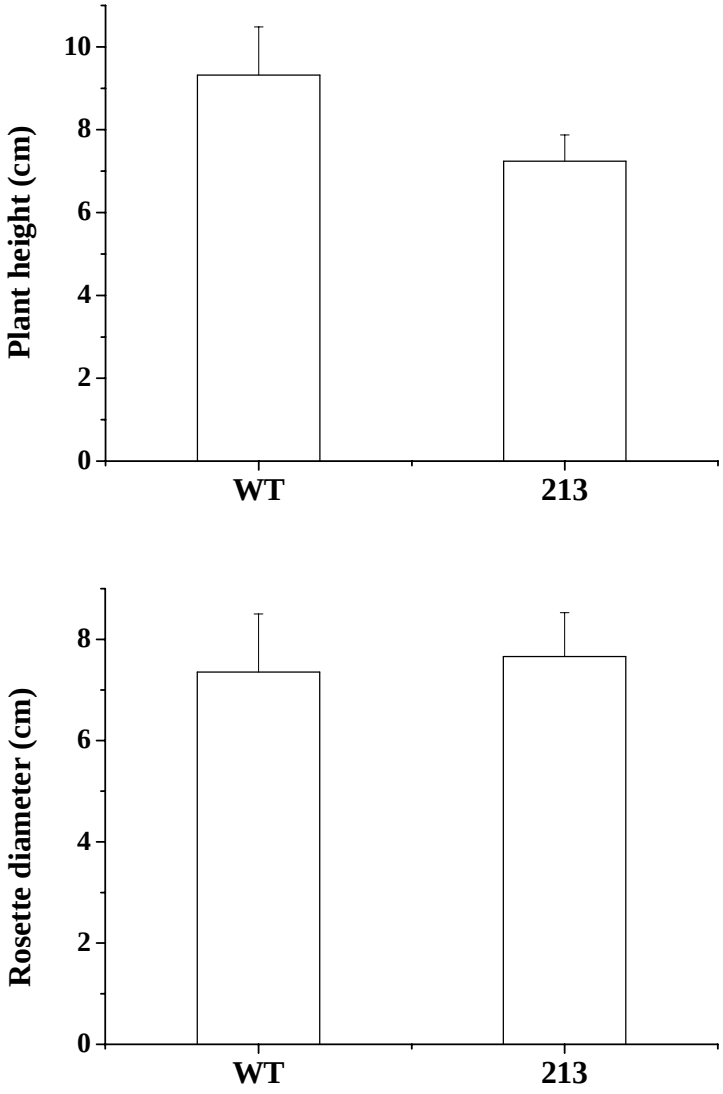


Figure S14. Growth of WT and ir-*npr1* line (213) grown in native populations in SW Utah. Plant height and rosette diameter was measured 25 days after transplanting (N=12).

Supplemental tables

Table S1. Pathogen identification from infected field samples. Pathogen strains were isolated from infected ir-*npr1* (213) field samples. Genomic DNA was isolated and the 16S rRNA was sequenced from each isolate. Identities were based on the homology and percent identity of known bacterial sequences in NCBI database. (Source: AMODIA, Braunschweig, customer number- **07002).**

Sample name	**Identification**	**Base pair matched**	**Percent identity**	**Pathogenicity**
Species 4	*Pseudomonas spps*	831 out of 835	99.5	pathogenic
Species 4a	*Pseudomonas jessenii*	826 out of 835	98.9	non pathogenic
Species 5	*Pantoea agglomerans*	405 out of 413	98.1	not tested

Table S2. Mean (± SE) volatile organic compounds (VOCs) emitted by OS-elicited single leaves of field-grown *N. attenuata* WT and ir-*npr1* (213) plants for 8 h. All compounds were analyzed in the same samples as those presented in Fig. 4C.

VOC compounds	**WT**	***ir-npr1***	*P*-value
(Z)-3-hexen-1-ol	31.92 ± 6.18	28.66 ± 8.24	0.644
(Z)-hexenyl-acetate	2.3 ± 0.62	1.73 ± 0.34	0.421
limonene	1.82 ± 0.51	1.66 ± 0.82	0.912
β-pinene	1.28 ± 0.02	1.76 ± 0.05	0.314
cis-jasmonol	13.15 ± 2.63	8.96± 1.28	0.066
Germacrene-A	1.22 ± 0.31	1.36 ± 0.24	0.088

Discussion

The main objectives of my PhD work were to study 1) the LOX3-JA-dependent responses in terms of insects' nutrition and 2) the importance of NPR1-dependent SA signalling in plant-herbivore interactions. To accomplish these goals, experiments were conducted on *N. attenuata* using the herbivores *M. sexta* (specialist) and *S. exigua* (generalist). The main tools in this study were *LOX3*- and *NPR1*-silenced plants. Experiments were conducted both under controlled glasshouse conditions and in nature, where *N. attenuata* grows and interacts with its natural herbivore community. For the first part of my work, I used a Waldbauer assay to measure insect growth performances, and correlated changes in insect performances with changes in *LOX3*-silenced plants. For the second part of my work, I analyzed the effect of silencing *NPR1* on phytohormone levels, defense metabolite accumulation, and gene expression profiles in *N. attenuata*. I tried to correlate how these molecular events affect JA-dependent IR in *N. attenuata.*

Genetic manipulation of genes involved in SA and JA biosynthesis: Advantages

To accomplish both the objectives of this study I used plants that were genetically manipulated for their gene expression. Since the genetically manipulated plants are isogenic toWT plants, excluding the gene which is under investigation we were successful in characterizing the molecular and ecological roles of the *LOX3* and *NPR1*. Studies with mutants defective in JA production have demonstrated the importance of genetic manipulation in plants for studying JA signalling (Halitschke and Baldwin, 2004) and pathogen attack (Thomma, Eggermont et al. 1998; Pieterse and Van Loon 2004). For example, a constitutive JA signalling-activated mutant, cev1, displays enhanced resistance to aphids, and to fungal and bacterial pathogens (Ellis et al. 2002), demonstrating the importance of JA signaling and gene manipulation to study plant resistance against pathogens. Therefore, to pursue our initial objectives, we used plants that were genetically manipulated in the expression of their *LOX3* and *NPR1* genes. This approach is particularly useful owing to the disadvantages in some previous studies wherein chemicals (SA and JA) were used to induce IR and link plant responses to insect performance. Although chemical treatment induces some components of IR, these treatments do not mimic actual herbivore responses. Moreover, herbiovore attack is known to activate multiple signaling pathways simultaneously in a time-dependent manner; these pathways in turn interact in a very complex manner to give responses that are quite different from chemical elicitation. Different pathways interact either antagonistically or synergistically, and such interaction helps plants regulate an appropriate combination of

defenses against a specific herbivore or pathogen. My study used transgenic plants that were defective in genes that had previously been implicated in SA and JA biosynthesis. In addition, instead of chemical elicitation of IR, I used techniques that closely mimic herbivore-related defense responses in plants.

I) Effects of LOX3-defenses on *M. sexta*'s nutritional physiology

Halitschke and Baldwin (2003) characterized *LOX3*-silenced plants in *N. attenuata.* These plants were impaired in their ability to accumulate JA and as a result, compared to WT plants, had lower levels of the direct defense metabolite nicotine and of those known collectively as protease inhibitors. Lower levels of direct defense compounds correlated with improved growth and development in *M. sexta.* We used Waldbauer nutritional indices to verify a) the mechanisms plants use to keep the growth and development of larvae under control and b) the counter-responses the insects employ to combat plant defense and avoid being eliminated by their host. We found that LOX3-JA-dependent defenses did not reduce the larval body mass of first-instar larvae but affected approximate digestibility (AD) of larvae. Contrary to the expectations, larvae feeding on *LOX3*-silenced plants showed improved AD. Such a finding is interesting given that larvae that fed on both WT and *LOX3*-silenced plants consumed the same amount of food in the early instars. The increased AD in the early instars of larvae that fed on *LOX3*-silenced plants greatly improved the consumption rates in their later stages of growth. As a result, the total leaf intake in second-instar larvae increased dramatically along with efficiency of conversion of digested food (ECD), the parameter that measures the efficiency with which larvae convert digested food into body mass. One significant finding from this study was the fact that nutritional changes in the early instars imposed by LOX3-JA-dependent defense metabolites can have a profound effect on the larvae in its late instars.

This study revealed that larvae at different instars respond differently to LOX3-JA-dependent defenses. During early instars, LOX3-dependent responses affect the ability of larvae to digest what they consume, which affects ECD in later instars. ECD and larval mass gain are functionally linked because later-instar larvae that fed on *LOX3*-silenced plants were significantly bigger and consumed more leaf mass than those that fed on WT plants. However, it is hard to prove whether increased ECD in the late instar larvae feeding on *LOX3*-silenced plants motivated them to consume more food or vice versa. But we strongly believe that the increased AD in the early instars has a positive effect on the ECD as the larvae develop which probably might help the late instar larvae to consume more food. This could be

true because the early instar larvae feeding on WT plants started with a low AD (due to the presence of LOX3-JA-dependent defenses metabolites) and although their AD slightly improved as they moved to late instar, but the body mass, total leaf consumption and ECD were significantly lower than those feeding on *LOX3*-silenced plants. Moreover, if we analyses the nutritional changes in the late instar larvae alone, it was quite surprising that the AD was not different between larvae feeding on WT and *LOX3*-silenced plants but ECD was. It could be likely that changes in AD might be obscured in larvae that fed on *LOX3*-silenced plants as they progress into late instars. Therefore, increased ECD in late instars feeding on *LOX3*-silenced plants might be a consequence of the increased AD in early instars. Larvae in early instars cannot consume more food (due to under-developed mouth parts); the only way they can improve the efficiency of ingested food is by increasing the digestibility of the food they consume. Therefore, it seems very logical for LOX3-JA-dependent defenses metabolites to affect AD particularly in the early insars. In desert locusts (*Schislocerca gregaria*), a similar trend (decreasing AD and increasing ECD) was observed as they moved from early to late instars which can be explained by shifts in food selection, digestive physiology, metabolic rates, and body composition (Lindroth, 1993).

What advantages can a *N. attenuata* derive by reducing the AD in early instars, and reducing ECD and total food intake in the late instars? One obvious reason is to slow down the growth and development of an herbivore. Keeping the larvae small has many advantages for *N. attenuata*. Larvae with small appetites and poor digestive ability consume less leaf area and have higher mortality rates, small larvae are preferred by predators. Also when larvae consume less its likely that larvae sequester less toxic compounds, which can be detrimental for the predators. But from a insect point of view, there is a counter-response; larvae of all instars increase their consumption index CI (the parameter that measures the amount of food ingested relative to the body mass gain) whenever ECD or AD is decreased. Usually larvae confined to a low-quality plant try to compensate for diminished food quality by consuming more of a plant they might otherwise avoid (Price et al., 1980). Then why is this compensatory feeding by larvae (feeding on WT plants) does not increase their body mass? One plausible explanation could be that the increased intake of diminished food quality (WT) rather than benefiting the larvae increases their exposure to defense compounds, which must subsequently be detoxified. Detoxification uses much of the energy that would otherwise be allotted to body mass, and as a result, larval ECD and body mass decreases. This is in line with earlier studies where they report that increased intake of low quality food can incur a lot of physiological and ecological costs (Lindroth, 1993). Certainly, it seems very likely that the

LOX3-JA-dependent defenses metabolites incur such costs in larvae which are reflected in the reduced larval ECD. The larvae always risk themselves by consuming more of the low quality foods. One way of reducing such risk would be to consume a sub-optimal dose of low quality, which the herbivores might learn during their adaptation process. These results support our view and provide evidence that there are indeed physiological and behavioural counter-responses by the herbivore towards LOX3-JA-dependent defenses metabolites in *N. attenuata*. But the physiological and ecological costs of detoxification are higher than the benfits the larvae gain from such a counter-response. Thus, LOX3-JA-dependent defenses metabolites of *N. attenuata* prove to be effective in resisting *Manduca sexta.*

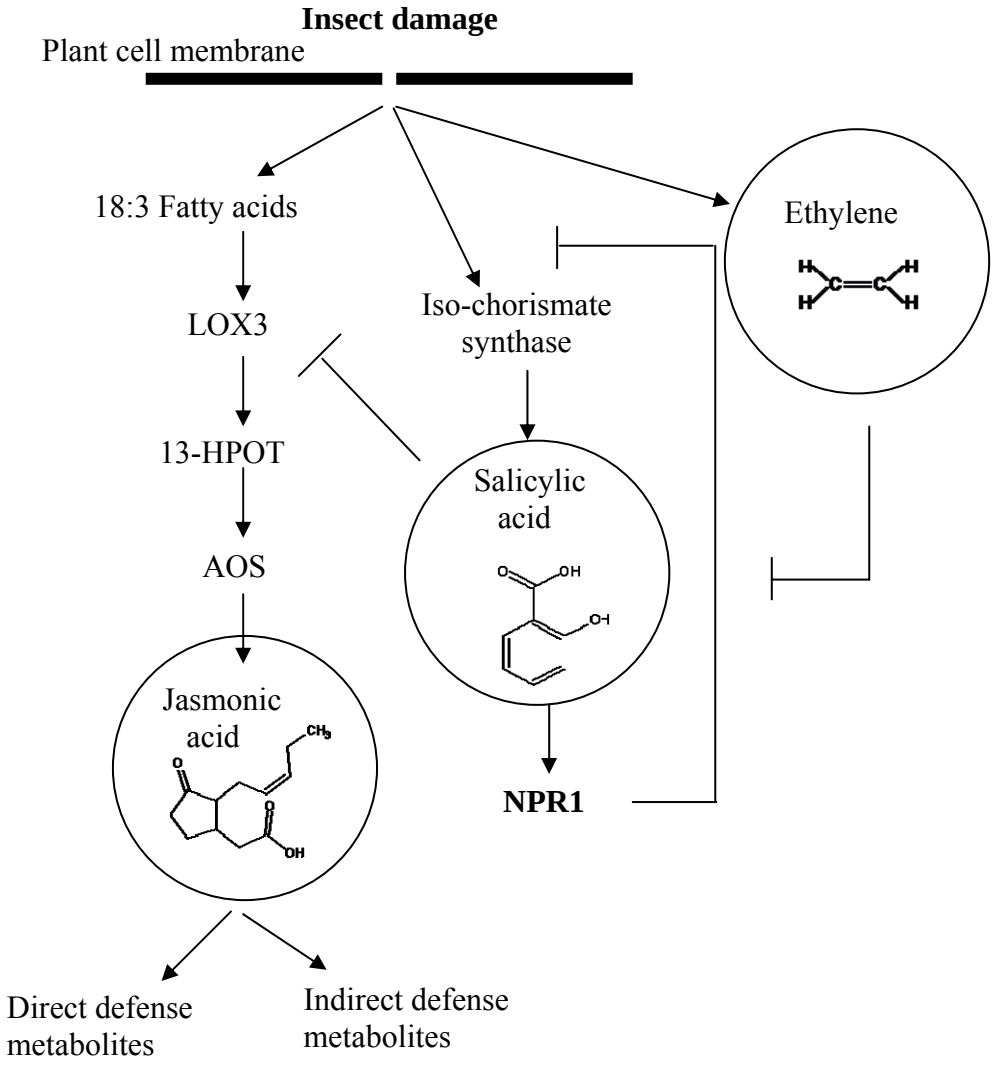
II) Importance of NPR1 responses in plant-herbivore interaction

The mutually antagonism sometimes seen in JA and SA pathways could help the plant to fine-tune the induction of its defenses in response to different herbivores. Therefore, simultaneous activation of multiple pathways in plants in response to a biotic stress may be a rule rather than an exception. However, our understanding of the SA-NPR1-dependent signalling pathways and their influence on LOX3-JA-dependent signalling pathway is limited. The second part of the study (Manuscript II) addresses exactly this. I used genetic tools to silence the expression of *NPR1*, and investigate the response of *NPR1*-silenced plants to herbivores in natural and glasshouse conditions and understand the role of NPR1 in fine-tuning defense responses (between SA and JA pathways) against herbivores.

NPR1 is a central player in systemic acquired resistance (SAR) where, it can regulate defense responses to pathogens. But NPR1-silenced *N. attenuata* lines planted in nature became susceptible to biotropic bacteria, *Pseudomonas sps* as well as generalist herbivores. This prompted us to study the role of NPR1 against herbivores. During pathogen attack, NPR1 is activated by SA to induces the expression of a range of pathogenesis-related (*PR*) genes such as *PR1*, *β-1, 3 glucanase* (*PR2*), and *PR5*, which confer resistance (Ward et al. 1991). Surprisingly, even after herbivore damage (*Spodoptera exigua*), *NPR1* is induced along with JA and SA. Since we know well regarding the importance of LOX3-JA-dependent defenses against herbivores, it was equally important to study in detail the role of SA and its effect on herbivore resistance. Under glasshouse conditions, *Spodoptera exigua*, performed best on *NPR1*-silenced lines. Therefore, we assumed that herbivores and pathogens seem to be inducing different responses using a common NPR1 in *N. attenuata*. This was indeed true, because NPR1 is a single copy gene in *N. attenuata*. As stated earlier herbivore attack induces

JA and SA production while pathogen attack induces SA production. Moreover, among the different NPRs studied to date in *Arabidopsis*, Na-NPR1 is most similar to *Arabidopsis thaliana* NPR1 (At-NPR1). At-NPR1 is well known for its role in pathogen defense. Therefore, we strongly believed that the role of NPR1 in IR can be better explained with reference to SA.

To a great extent, NPR1-silenced lines behave like *LOX3*-silenced lines because JA accumulation in both genotypes is impaired. Consistent with *LOX3*-silenced plants, the greater susceptibility of *NPR1*-silenced plants to herbivores could be attributed to their impaired ability to elicit indirect (*cis*-α-bergamotene release) and direct (nicotine) defense responses. As a result, *NPR1*-silenced plants are less able to attract *Geocorus pallens* predators and less able to increase nicotine levels. Both predator attraction and nicotine accumulation are known to be elicited by JA signaling. But the unique feature of *NPR1*-silenced plants is to accumulate high levels of SA and low levels of JA in response to herbivore attack and OS elicitation. One hypothetical reason for the low JA in ir-*npr1* plants could be due to the presence of high SA. This is a plausible explanation given the antagonistic nature of SA and JA towards each other. For example, the eicosanoids of animals, which are derivatives of C20:4 fatty acids share biosynthetic and structural similarities with the jasmonates (which are synthesized from 18:3 fatty acids). The cyclooxygenase enzymes of animals, like the LOXs of plants, are inhibited by salicylates, the best studied of which is acetylsalicylate (aspirin) (Vane, 1971). We tested this hypothesis by exogenous JA application to nicotine-deficient ir-*npr1* plants. As expected, normal nicotine levels and resistance to *S. exigua* was restored in *NPR1*-silenced plants. Moreover, exogenous SA increased the susceptibility of WT plants like those of untreated *NPR1*-silenced plants. Therefore, it is evident that the increased SA in field- and glasshouse-grown ir-*npr1* plants which accumulate high levels of SA can inhibit JA and its dependent responses, thereby increasing the susceptibility to herbivore. Plants such as *N. attenuata* seem to have evolved sophisticated means of resisting pathogens and herbivores using common regulatory genes such as NPR1 which can help plants fine tune their defense responses. One such example of fine tuned responses is controlling SA production during herbivory. Uncontrolled SA production in plants can have many side effects apart from inhibiting the JA pathway. For example., high SA levels are also associated with stunted growth (Mauch, Mauch-Mani et al. 2001; Shah 2003). Consistent with this study, our results also show a down regulation of *RUBISCO* and *PSII* transcripts in *NPR1*-silenced plants. It seems likely that NPR1 has position itself well to regulate primary and secondary metabolism during herbivory.



A model summarising the role of NPR1 in IR. Insect damage results in a increased production of three phytohormones (Jasmonic acid, Salicylic acid and ethylene). Jasmonic acid is known to mediate the prodcution of various direct and indirect secondary defense metabolites. Given the nature of Salictlic acid to inhibit LOX3 and subsequently JA, NPR1 serves the role of a negulator regulator of saliylic acid in plants. Controlled SA is vital for an unfettered elicitation of JA-dependent responses. Apart from NPR1, ethylene also seemd to negative regulate SA production in *N. attenuata*. Blunt arrows indicate negative regulation/inhibition and arrow indicate postive regulation. Signaling crosstalk between Jasmonic acid, Salicylic acid and ethylene, provides *N. attenuata* the much needed regulatory signals (by positive and negative feedvbacks) by which IR is optimized.

But how NPR1 responds differently to herbivore- and pathogen-related elicitors remain a question. Perhaps the promoter region of known *NPR1* has a W box consensus sequence that

can bind WRKY transcription factors. Both herbivores and pathogens are known to stimulate WRKYs (Yu et al. 2001; Qu and Baldwin, 2007, unpublished); and WRKYs could be the mediators. The second possibility involves SA induction and its associated redox potential changes in a plant cell (Vanacker, Lu et al. 2001). NPR1 has several cysteins residues which could be the target of redox regulation. In support of this, we observe increased SA production in *NPR1*-silenced plants. Since the expression of NPR1 (gene and protein) after *S. exigua* attack in WT is increased compared to constitutive levels in uninduced plants, SA and the redox changes in plants could be responsible. Finally, it is also possible that some pathogenic factors in the oral secretions of the herbivore elicit SA and NPR1, similar to what is observed during pathogen infection.

Herbivore attack elicits an oxidative burst, and based on these observations a link may be established between SA and NPR1 in *N. attenuata.* These results are consistent with the view that generalist herbivores, such as *S. littoralis*, may activate the SA pathway concomitantly with the JA pathway, perhaps to weaken JA-mediated resistance by amplifying the SA-JA antagonism (Stotz *et al.,* 2002; Cipollini *et al.,* 2004). We propose that the initial SA production stimulates NPR1 activity and that the stimulated NPR1 then deregulates SA biosynthesis. The SA that is increased after NPR1 silencing negatively influences the outcome of direct and indirect defenses. This controlled SA production by NPR1, seems vital for a plant to elicit IR owing to the chemical nature of SA to inhibit JA. There are several studies which support the view that controlled SA production is vital for proper induction of IR. For example, in *Nicotiana sylvestris*, MeSA application reduces elicited nicotine accumulation (Baldwin et al. 1996; Baldwin et al. 1997). Several studies in *Nicotiana* species, have reported that SA can antagonize JA- and its mediated defenses. In *Nicotiana tabacum,* TMV-inoculated plants (which are associated with local and systemic increase in endogenous SA) attenuated wound-induced JA and nicotine responses. As a result, larvae consumed 1.7 to 2.7 times more leaf tissue from TMV-inoculated plants than from mock-inoculated plants (Preston et al. 1999). Therefore, the role of NPR1 in regulating SA production during IR seems to be justified.

Summary

Induced resistance to herbivores is a complex trait and involves interactions between multiple signalling pathways. Synergistic and antagonistic relations between pathways help the plant to regulate a defense that is appropriate to a particular herbivore. JA-dependent *LOX3* responses and SA-dependent NPR1 responses are two important signalling pathways herbivores use to regulate their defenses. JA-dependent *LOX3* responses are well-studied and known to be vital in mounting plant resistance. Two aspects that are unexplored are 1) the impact of JA-dependent *LOX3* responses on herbivores' nutritional physiology and 2) the influence of SA-dependent NPR1 responses on JA-dependent *LOX3* responses in plant-herbivore interactions.

We used Waldbauer nutritional indices to measure the pre- and post-ingestive effects of JA-dependent *LOX3* responses on insects' nutritional physiology. *LOX3*-mediated defenses reduced larval growth, consumption, and frass production. These defenses reduced how efficiently late-instar larvae converted digested food to body mass (ECD). In contrast, *LOX3*-mediated defenses decreased the approximate digestive ability (AD) of early-instar larvae without affecting their ECD and total food consumption. Larvae that fed on defense-elicited WT plants altered their behavior by consuming more food per unit of body mass gain compared to those that fed on *LOX3*-silenced plants. We suggest that the changes that occur in early-instar larvae are crucial for determining behavioral responses in late-instar larvae. JA-dependent *LOX3* responses decreased the AD of early-instar larvae, preventing them from consuming more food in the later stages and consequently decreasing both their ECD and the efficiency of conversion of ingested food. JA-dependent *LOX3* responses represent stringent behavioral and physiological counter-responses of insects to plant defenses.

Herbivore attack on *N. attenuata* elicits SA and the regulatory gene *NPR1* along with its well-known *LOX3*-JA-dependent direct and indirect defenses. In *N. attenuata* silencing NPR1 reduced resistance to herbivores and pathogens in nature and to *S. exigua* under glasshouse conditions. We could correlate lowered resistance with the reduced accumulation of JA-elicited direct defense metabolites (nicotine, caffeoyl putrescine, and rutin). In addition, *NPR1*-silencing reduced the ability of *N. attenuata* to attract the predators (*G. pallens*) of plants' herbivores, which could be correlated with the low emission of a volatile organic compound (*cis*-α-bergamotene). NPR1-silenced plants consistently accumulated high levels of free SA in response to herbivory or herbivores' oral secretions. Typically, *NPR1*-silencing affected JA-dependent *LOX3* responses, which could be correlated with increased SA. NPR1-deregulated SA could be traced to the increased transcript

accumulation of isochorismate synthase (involved in SA production) and the reduced transcript accumulation of *LOX3*. Exogenous JA application in *NPR1*-silenced plants restored normal nicotine leves and also resistance to generalist herbivore *S. exigua*. On the contrary, SA application increased the susceptibility of *N. attenuata'*s WT plants to *S. exigua* similar to untreated *NPR1*-silenced plants. Cleary, the loss of resistance to *S. exigua* in *NPR1*-silenced plants seems to be due to SA antagonizing the JA responses. Since, JA-dependent *LOX3* responses and SA-dependent NPR1 responses in *N. attenuata* were activated simultaneously. We conclude that NPR1 serves as a negative regulator of SA biosynthesis in response to herbivory and in order to activate the JA pathway, and helps the plant fine-tune an appropriate defense response to herbivores.

Zusammenfassung

Pflanzen haben verschiedene konstitutive und induzierbare Mechanismen entwickelt, um Herbivore abzuwehren. Induzierbare Abwehr stellt einen phenotypisch-flexiblen Mechanismus dar, da die Pflanze ihn nur bei Herbivorenbefall aktiviert. Die Signalwege, die zur induzierten Resistenz führen, sind komplex und involvieren verschiedenste Signalmoleküle. Bei Herbivorenbefall aktivieren Pflanzen hauptsächlich Jasmonsäure- und Salizylsäure-Signalkaskaden. Synergistische und antagonistische Interaktionen dieser Signaltransduktionswege sind bekannt und es wird postuliert, dass die Integration mehrerer Signalwege es der Pflanze ermöglicht, ihre Reaktionen auf spezifische Herbivore zuzuschneidern. Die Gene Lipoxygenase 3 (LOX 3) und Nonexpressor of PR1 (NPR1) spielen eine wichtige Rolle im Jasmonsäure- bzw. Salizylsäure-Signalweg. LOX 3 kodiert für ein Enzym der Jasmonsäure-Biosynthese und Jasmonsäure-abhängige Prozesse sind in Pflanzen unerlässlich für die Produktion von direkt der Herbivorenabwehr dienenden Sekundärmetaboliten wie Proteinaseinhibitoren und Alkaloiden. Desweiteren ist die Jasmonsäure-Kaskade wichtig für indirekte Abwehrmechanismen wie die Emission flüchtiger organischer Verbindungen (VOCs), die in tritrophischen Interaktionen zwischen Pflanze, Herbivoren und Prädatoren eine Rolle spielen können. NPR1 ist verantwortlich für die Aktivierung von Salizylsäure-abhängigen Resistenzgenen und deshalb notwendig für die Abwehr der Pflanze gegen Pathogene.

Mehrere Aspekte, die die Funktion von LOX 3 und NPR1 in Jasmonsäure- und Salizylsäure-Signalnetzwerken angehen, sind ungeklärt. Zwei wichtige Punkte sind (1) der Effekt von LOX 3-vermittelten Reaktionen der Pflanze auf die Ernährungsphysiologie der Herbivoren und (2) der Effekt von NPR1-abhängiger Genexpression auf den Jasmonsäure-Signalweg.

In der vorliegenden Arbeit wurde der wilde Tabak *Nicotiana attanuata* zusammen mit seinem natürlichen Herbivor *Manduca sexta* als Modell genutzt, um das Zusammenspiel von Jasmonsäure- und Salizylsäure-Signalwegen in induzierter Pflanzenabwehr zu erforschen. Die Ergebnisse sind im Folgenden schlaglichtartig zusammengefasst.

1) **LOX -vermittelte Jasmonsäure-Netzwerke in *N. attenuata* und ihr Effekt auf die Ernährungsphysiologie und das Verhalten von *M. sexta***

   • LOX 3-bediongte Abwehrreaktionen beeinflussen den Ernährungsstatus von *M. sexta* während verschiedener Larvalstadien. Bei Vergleich der Nahrungs-aufnahme von *M. sexta* Raupen auf Wildtyp-Pflanzen (WT) und auf transgenen Linien (as*LOX3*) mit veringerter Expression von LOX 3 wurde festgestellt, dass Raupen in frühen

Larvalstadien zwar zunächst ähnliche Mengen Blattmasse konsumierten, dass sie aber in späten Larvalstadien auf WT-Pfanzen deutlich weniger fraßen als auf as*LOX3*-Pflanzen.

- LOX 3-betroffene Abwehrreaktionen reduzieren die Verdaubarkeit (approximate digestibility, AD) des Pflanzenmaterials in frühen *M. sexta* Larvalstadien. Als Folge ist die Retention von Nährstoffen durch junge Larven beeinträchtigt. Ein verringerte AD beeinflusst die Gesamtnahrungsaufnahme in späteren Larvalstadien.
- Die Effizienz, mit der konsumierte Nahrung in Körpermasse umgewandelt wird, (efficiency of conversion of digested food, ECD) und die Gesamtnahrungsaufnahme sind reduziert in späten Larvalstadien durch LOX 3-abhänginge Prozesse. Dafür verantwortlich sind hauptsächlich Abwehrmetaboliten der Pflanze, deren Biosynthese durch Jasmonäure induziert wird.
- *M. sexta* Raupen versuchen die verringerte AD in frühen Larvalstadien und verringerte ECD in späten Larvalstadien auszugleichen, indem sie die Rate von aufgenommener Nahrung zu Körpergewichtzunahme (consumption index, CI) erhöhen. Jedoch kommt eine erhöhte CI den Raupen nicht zugute, da sie dadurch ebenfalls höhere Mengen an giftigen Pflanzenabwehrstoffen zu sich nehmen. Dadurch sind die Kosten der Detoxifikation höher, die wiederum die Gewichtszunahme der Raupe beeinträchtigen.

2) **Der Einfluss von NPR1-abhängigen Salizylsäure-Signalen auf LOX 3-vermittelte Abwehrreaktionen in *N. attenuata***

- Transgene *N. attenuata* Pflanzen mit verringerter NPR1-Expression sind anfälliger für Herbivorie und Pathogene (*Pseudmonas* spp.) unter natürlichen Bedingungen und unter Gewachshausbedingungen.
- Die im Vergleich zu WT-Pflanzen erhöhte Anfälligkeit ist korreliert mit verringerter Akkumulation von Abwehrstoffen wie Nikotin, Caffeoylputrescin und Rutin, die in Jasmonsäure-abhängiger Weise produziert werden.
- Pflanzen mit verringerter NPR1-Expression ziehen weniger Prädatoren von Herbivoren an. Diese Tatsache ist auf die verringerte Produktion der Anlockungssubstanz *cis*-α-Bergamoten zurückzuführen, dessen Biosynthese auch von Jasmonsäure abhängt.
- Pflanzen mit verringerter NPR1-Expression akkumulieren bei Herbivorenbefall höhere Mengen an Salizylsäure als WT-Pflanzen. Daher ist anzunehmen, dass NPR1 als negativer Regulator der Salizylsäure-Produktion nach Herbivorenbefall wirkt.
- Erhöhte Salizylsäure-Produktion in Pflanzen mit verringerter NPR1-Expression führt zu reduzierter Expression von LOX 3 und geringerer Jasmonsäure-Produktion. Letzteres

wiederum verringert direkte und indirekte Abwehrreaktionen von *N. attenuata* auf Herbivorie.

- NPR1 ist ein zentraler Integrationspunkt von Signalwegen, die in Abwehrreaktionen der Pflanze involviert sind. NPR1 trägt in *N. attenuata* zur Regulation der Salizylsäure-Produktion bei und vermindert damit den antagonistischen Effekt von Salizylsäure auf den Jasmonsäure-Signalweg.
- Exogen applizierte Jasmonsäure erhöht die Nikotinkonzentration und damit die Resistenz gegen *S. exigua* in transgenen *NPR1*- Pflanzen. Werden dagegen Wt-Pflanzen mit SA behandelt, dann sinkt deren Resistenz gegen *S. exigua* auf das Niveau von *NPR1*-Pflanzen. Dises Experimente verdeutlichen, dass die erhöhte Anfälligkeit von Pflanzen mit reduzierten *NPR1*-Transkripten für *S. exigua* ein Resultat der antagonistischen Wirkung von SA auf die JA-vermittelte Verteidingsreaktion ist.

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8.5 **Declaration of Independent Assignment**

I hereby declare that in accordance with the conferral of doctrol degree from the School of Biology and Pharmacy of Friedrich Schiller University, Jena that the submitted thesis was written only with the assistance and literature cited in the text.

I was not assisted by any consultant for my doctorate thesis. The People who helped in the experiments, data analysis and writing the manuscripts are acknowledged in the respective manuscripts.

The thesis has not been previosly submitted either to the Friedrich Schiller University, Jena or to any other University

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